Rhabdovirus-induced microribonucleic acids in rainbow trout (Oncorhynchus mykiss Walbaum)

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Rhabdovirus-induced microribonucleic acids in rainbow trout (*Oncorhynchus mykiss* Walbaum)

PhD Thesis

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PREFACE

This thesis is submitted in partial fulfillment of the requirements for the Doctor of Philosophy (PhD) degree at the Technical University of Denmark. The work was carried out at the Fish Health Section, Department of Animal Science, Aarhus University (formerly the Fish Host-Pathogen Interactions Laboratory, National Veterinary Institute, Technical University of Denmark), under the supervision of Dr. Niels Lorenzen (main supervisor) and Dr. Brian Dall Schyth (co-supervisor). The project was funded by the Danish Technical Research Council Grant 247-08-0530 (Co-evolution), the European Economic Community (EEC) Sixth Framework Programme (Food Quality and Safety) Improved Immunity of Aquacultured Animals (IMAQUANIM) Contract No. 007103, and the European Union Network of Excellence EPIZONE Contract No. FOOD-CT-2006-016236.

The thesis is based on studies on microRNA expression profiles in a teleost fish in the contexts of lethal rhabdovirus infection and vaccination with a DNA vaccine encoding an antigenic protein of the virus. Chapter 1 briefly introduces the thesis and describes its objectives. Background information is presented in Chapter 2. The major studies are described in Manuscript 1 and Manuscript 2. A review paper, Manuscript 3, is likewise included.

ACKNOWLEDGMENT

Foremost, I would to express my profound gratitude to my adviser, Professor Dr. Niels Lorenzen for providing me the opportunity to pursue my studies, for giving me his trust to work on the research project under his tutelage, and for his unwavering support. I thank my co-supervisor Dr. Brian Dall Schyth for his guidance and fruitful discussions.

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Finally, I give thanks to my family and friends in the Philippines and elsewhere around the world for the love and support across the miles. I thank my friends in Aarhus for the camaraderie which made my stay in Denmark enjoyable and the best experience that it can be.

LIST OF ABBREVIATIONS

Ago	Argonaute protein
BF-2	Bluegill fry – fish cell line
CMV	Cytomegalovirus
DALRD3	DALR anticodon binding domain containing 3
DGCR8	DiGeorge syndrome critical region gene 8
DNA	Deoxyribonucleic acid
EPC	*Epithelioma papulusom cyprinid* – fish cell line
ELISA	Enzyme-linked immunosorbent assay
GAS	Gamma interferon activated site
HeLa	Henrietta Lacks (human cell line)
HEK293T	Human embryonic kidney cell line
IFN	Interferon
IMPDH2	Inosine monophosphate dehydrogenase 2
ISRE	Interferon-stimulated response element
miRNA	Microribonucleic acid
PHA	Phytohemagglutin
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
TLR	Toll-like receptor
UTR	Untranslated region
VHS	Viral hemorrhagic septicemia
VHSV	*Viral hemorrhagic septicemia virus*

SUMMARY

This thesis deals with microribonucleic acid (microRNA; miRNA) expression during rhabdovirus infection and upon immunization with a DNA vaccine expressing the rhabdovirus glycoprotein in teleost fish. MicroRNAs are conserved, small, endogenous, non-coding regulatory RNAs that modulate gene expression at the post-transcriptional level. The genes whose expression they control are involved in numerous aspects of an organism's biology in which abnormal miRNA expression is associated with pathologies.

In this thesis, the upregulation of two clustered miRNAs was observed in rainbow trout infected with the fish rhabdovirus, viral hemorrhagic septicemia virus (VHSV) and in fish immunized with a DNA vaccine encoding the glycoprotein of VHSV. It was shown that the two miRNAs, known so far only in teleost fishes, are orthologues of an ancient vertebrate miRNA cluster, which in humans are involved in the regulation of the cell cycle and have been associated with various types of cancers. Interferon (IFN)-related regulatory sequences were found in the promoter of the teleost fish miRNA cluster and its expression was induced by IFNs and IFN-related mechanisms. It was further demonstrated that these teleost miRNAs may participate in the IFN-mediated antiviral response.

IFN-induced miRNAs contributing to the antiviral effects of type I IFN have been demonstrated and an increasing number of cellular miRNAs have been shown to be involved in the antiviral response against different viruses in mammalian cells. The findings in this thesis represent the first report to address the expression of particular miRNAs in response to rhabdovirus infection and to anti-rhabdovirus DNA vaccination in teleost fish, as well as the first teleost fish IFN-elicited miRNAs that could potentially be involved in teleost immune responses. Thus, RNA interference mechanism mediated by cellular miRNAs might play an important role in innate antiviral immune responses in teleost fish. An update on the antiviral roles of miRNAs in mammalian cells and potentially in vertebrate cells is included in a review paper as part of the thesis.

SAMMENDRAG

Nyere forskning har vist, at reguleringen af hvor kraftigt gener i dyr og menneskers celler udtrykkes ikke kun foregår i cellekernen. Man har således tidligere antaget at det især var i omskrivningen af cellekernens DNA til budbringer RNA (messenger RNA eller mRNA) at geners aktivitet blev styret. Det har imidlertid vist sig at små RNA molekyler, kendt som mikroRNA, ude i cellens cytoplasma kan parre sig med bestemte mRNA molekyler og dermed blokere for oversættelsen af disse til protein. Mekanismen, der betegnes RNA-interferens er meget velbevaret op igennem dyreriget og bruges ikke kun til at regulere kroppens egne gener, men også som forsvar mod fx virusinfektioner. I dette projekt har fokus været at analysere og karakterisere regnbueørredens mikroRNA-reaktion på infektion med en smitsom virus. To mikroRNA molekyler, som tidligere har været beskrevet hos blandt andet zebrafisk, blev meget kraftigt aktiveret. Nærmere analyse af deres regulering viste at blandt andet interferon, som er kroppens vigtigste akutte forsvarskomponent mod virusinfektioner, styrer aktiveringen af de to RNA molekyler. Selvom de to mikroRNA molekyler ikke direkte er beskrevet hos mennesker, viste en sammenlignende gensekvensanalyse at mennesker og andre varmblodede dyr besidder nogle meget tæt beslægtede, men mere originale varianter. Hos fisk er de to mikroRNA´er sandsynligvis blevet specialiserede til at indgå i forsvarsmekanismerne mod virusinfektioner. Forsøg hvor fisk blev smittet med virus efter aktivering af interferon viste nemlig at beskyttelsen mod virusinfektionen blev nedsat, når de to mikroRNA´er forinden blev blokeret. Resultaterne bidrager til vores samlede forståelse af hvordan dyr og mennesker forsvarer sig mod virusinfektioner og kan på sigt bidrage til udvikling af nye metoder til sygdomsforebyggelse og –behandling.

1 INTRODUCTION AND OBJECTIVES

Microribonucleic acids (microRNAs or miRNAs) are a class of evolutionarily conserved, short (roughly 22 nucleotides (nt) in length), endogenously expressed non-coding RNAs. MicroRNAs control the expression of genes post-transcriptionally by negatively regulating transcript levels or inhibiting protein synthesis (Bartel, 2004). They play crucial regulatory roles in multiple biological processes including development, metabolism, immune responses, and host-pathogen interactions. Dysregulated expression of miRNAs is linked to several pathological conditions, such as cancer, which underscores the essential functions of miRNAs in various normal physiological contexts.

Viral hemorrhagic septicemia virus (VHSV) is a rhabdovirus that causes viral hemorrhagic septicemia (VHS), infecting several freshwater and marine fish species. The virus was first identified from farmed rainbow trout (*Oncorhynchus mykiss*) in Denmark in 1965 and since then has been known to occur widely in the marine waters of the Northern Hemisphere. VHS has a major impact on commercial production of rainbow trout because of huge economic losses that result from very high mortality rates (Olesen, 1998).

The disease has long been eradicated in aquaculture farms in Denmark. Nevertheless, the existence of a wild marine reservoir of VHSV threatens the increasing production of rainbow trout in sea farms with potential VHS outbreaks. This necessitates improved disease management/prevention approaches in order to alleviate the impact of potential VHS outbreaks in rainbow trout marine production systems.

A DNA vaccine based on the glycoprotein G gene of VHSV has been demonstrated to protect fish from lethal infection under experimental conditions (Lorenzen and LaPatra, 2005; Lorenzen, 1998). The DNA vaccine stimulates an immune response that includes early, non-specific, and short-term protection, and subsequently by specific, long-term immunity (Kurath, 2008; Lorenzen and LaPatra, 2005). Even though the exact protective mechanism is not completely known, it is believed that non-specific and specific immune mechanisms complement each other to contribute to the high efficiency of the DNA vaccine (Lorenzen and LaPatra, 2005).

Immune responses involve cell activation, proliferation, and differentiation. During these cellular processes, cell phenotypes are altered, accompanied by gene expression changes such that genes characteristic of particular (immune) cell types are expressed. Gene expression is a highly regulated process and employs several mechanisms that operate at various levels. The discovery of miRNAs as major players in controlling gene expression at the post-transcriptional level adds to the molecular diversity of the gene regulatory machinery.

Therefore, miRNA expression profiles were investigated during lethal infection with VHSV in the teleost fish rainbow trout (*O. mykiss*) and in fish vaccinated with a DNA vaccine encoding the glycoprotein of VHSV.

Strong expression of a miRNA cluster comprising miR-462 and miR-731 (herein referred to as the miR-462/731 cluster), miRNAs which are described thus far only in teleost fishes, was observed in the liver of *O. mykiss* infected with VHSV. The presence of immunologically-relevant gene regulatory sequences found proximal to the miR-

462/-731 locus suggests the potential involvement of these two miRNAs in interferon (IFN)-mediated anti-viral immune responses. Furthermore, nucleotide sequence analysis indicates that miR-462 and miR-731 are orthologues, respectively, of miR-191 and miR-425 (herein referred to as the miR-425 cluster), which are ancestral miRNAs found in genomes of cartilaginous fish and higher vertebrates, including humans (Manuscript 1).

The miR-462 cluster was also found to be very highly induced in the skeletal muscle (site of vaccine administration) and the liver of *O. mykiss* injected with the VHSV glycoprotein gene-based DNA vaccine. The expression of miR-462 and miR-731 was elicited by both type I and type II IFNs in both the site of injection and liver in fish injected with plasmid constructs encoding IFN 1 and IFN-γ. Finally, antagonizing miR-462/731 with intraperitoneally-injected, saline-formulated anti-miR-462 and anti-miR-731 oligonucleotides in poly I:C-treated rainbow trout fingerlings followed by VHSV challenge reduced the protective effect of poly I:C and caused higher mortalities. By counteracting poly I:C-induced miR-462/-731 expression with specific synthetic inhibitory oligonucleotides, results indicate that the upregulation of these two miRNAs and their activities are involved in IFN-mediated protection of trout against VHSV (Manuscript 2).

RNA-based antiviral responses in which virus-derived small interfering (si-)RNAs are used by the RNA interference machinery to target and inhibit virus RNAs are well-established in invertebrates and plants. A similar mechanism has recently been shown to operate in a context-dependent manner in mammalian cells and in mice. It is also believed that mammalian cells (or potentially vertebrate cells) employ miRNAs in an

RNAi-based antiviral strategy. The involvement of miRNAs in antiviral responses in mammals has been the subject of intense research efforts in recent years. Inhibitory miRNAs that directly target virus RNAs and those that target host mRNAs involved in host-pathogen interactions and immune responses have been reported in numerous studies. To date, no teleost fish miRNA has been implicated in these processes, although many mammalian miRNAs with such functions have homologues in teleost fishes. Manuscript 3 updates on the current knowledge of RNA-based antiviral mechanisms in vertebrates.

2 BACKGROUND

This chapter reviews literature that provides fundamental scientific background knowledge pertaining to the experimental work described in the manuscripts contained herein.

2.1 Viral hemorrhagic septicemia virus

Viral hemorrhagic septicemia virus (VHSV) is a rhabdovirus (Gr. *rhabdos* = rod) with a bullet-shaped, enveloped virus particle (virion), possessing a negative sense single stranded RNA genome (Figure 2). It belongs to the genus *Novirhabdovirus* of the *Rhabdoviridae* family (ICTV, 2012; Van Regenmortel et al., 2000) and is the aetiological agent of the disease called viral hemorrhagic septicemia (VHS) (Jensen, 1965; Jørgensen, 1974). The novirhabdovirus genome comprises six open reading frames that codes for the following proteins: the nucleoprotein (N), polymerase-associated phosphoprotein (P), matrix protein (M), glycoprotein (G), non-virion protein (NV) and the large RNA-dependent RNA polymerase (L) protein (Walker et al., 2000; Schutze et al., 1999). The G protein has been shown to be the target of protective neutralizing antibodies (Lorenzen et al., 1999; Lorenzen et al., 1990).

Typical clinical signs in infected fish are the extensive hemorrhaging in external and internal organs that varies among fish species, ascites, exopthalmia, and skin darkening. Histopathological features include necrotic changes in the liver, spleen, hematopoietic tissue, and pancreatic acini in numerous fish species (Kurath and Winton, 2008; Isshiki et al, 2001).

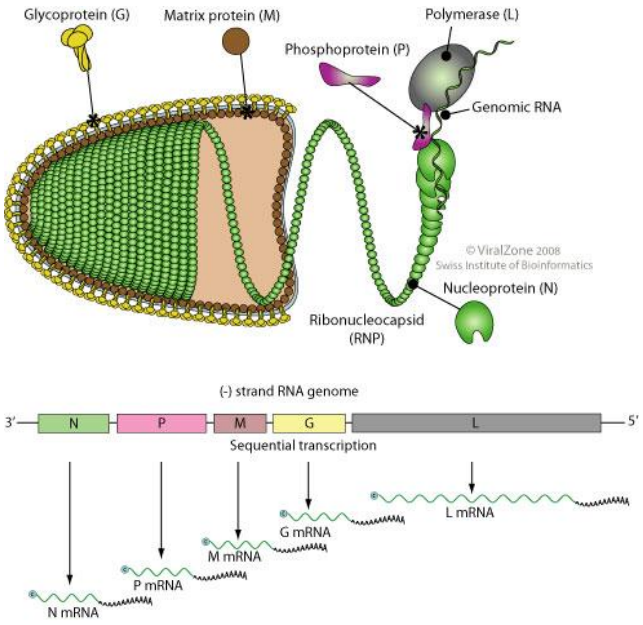

Figure 2. Schematic representation of rhabdovirus virion which is enveloped and bullet-shaped and contains a negative sense, single stranded RNA genome. The genome encodes both structural and non-structural proteins. (http://viralzone.expasy.org/all_by_species/2.html)

VHSV can infect numerous species of both freshwater and marine fish. The virus was first isolated and identified from farmed rainbow trout (*Oncorhynchus mykiss*) in Denmark in 1965(2?) (Jensen, 1965; Jørgensen, 1974) and was thought to infect only European freshwater salmonid species until 1988 (Einer-Jensen, 2013). To date, VHSV is known to occur widely in the marine environment, having been identified in the waters of North America (Hedrick et al., 2003), Japan (Takano et al., 2000), and continental Europe (Skall et al., 2005; Mortensen et al., 1999; Olesen, 1998). At least 28 species of wild fish in the North American Great Lakes are vulnerable to VHSV infection (USDA-APHIS, 2008a, b). VHS is a highly significant virus disease of salmonid fish in European aquaculture, with major impact on commercial production of

rainbow trout, resulting in up to 90% mortality (Olesen, 1998) causing massive economic loss. Apart from rainbow trout (Smail, 1999), VHS also seriously impacts culture of turbot (*Scophthalmus maximus*) (Ross et al., 1995), and Japanese flounder (*Paralichthys olivaceus*) (Isshiki et al, 2001).

It is believed that VHSV originated from marine waters (Dixon, 1999) and the virus may have changed hosts several times. A close genetic linkage between marine VHSV and rainbow trout VHSV from European farms has been demonstrated (Einer-Jensen et al., 2004). Likewise, data also indicated that adaptation of VHSV to rainbow trout has taken place a number of times in the past 50 years in European rainbow trout aquaculture (Einer-Jensen et al., 2004). Several studies also corroborate the hypothesis that the origin of VHSV is the marine environment (Pierce and Stepien, 2012; Thompson et al., 2011; Snow et al., 2004).

Four main VHSV genotypes (I, II, III, and IV) are recognized, which cluster based on geographical location instead of serotypes or host species (Pierce and Stepien, 2012; Einer-Jensen et al., 2004; Snow et al., 1999). Genotypes I-III are found in Europe while genotype IV is found in Asia (Japan and Korea) and North America (Einer-Jensen et al., 2004; Kim et al., 2011; Snow et al., 1999).

European freshwater strains consisting of rainbow trout-adapted VHSV belong to genotypes I and III (Einer-Jensen et al., 2004; Dale et al., 2009), whereas strains isolated from marine fish species have been assigned to the four genotypes. Marine VHSV strains either do not cause disease or are very weakly pathogenic to rainbow trout (Skall et al., 2004). Nonetheless, marine strains comprise the bulk of genetic

diversity (Einer-Jensen et al., 2004) and because single-stranded RNA (ssRNA) viruses like VHSV exhibit very high mutation rates (Domingo, 2000; Domingo and Holland, 1997), the prospect of evolving from non-pathogenic to pathogenic forms is extremely high, particularly when permitted contact with a new host (species barrier crossing).

Outbreaks of VHS have occurred in rainbow trout sea farms in Finland (Husu-Kallio & Suokko 2000; Raja-Halli *et al.* 2006), Sweden (Nordblom 1998; Nordblom & Norell 2000), and Norway (Dale *et al.* 2009). In these cases, it is very likely that VHSV from marine wild fish shifted hosts due to repeated introduction of marine VHSV strains into farmed rainbow trout.

VHS outbreak strains in Norway showed the closest genetic relatedness to marine VHSV isolates from neighboring marine waters (Einer-Jensen et al., 2004; Dale et al., 2009), as were the Finnish and Swedish isolates. Such outbreaks are most probably due to host changes from wild marine fish and adaptation to farmed rainbow trout (Schonherz et al., 2012). This indicates that non- or less pathogenic marine VHSV strains can give rise to new pathogenic strains and that the marine environment harbors a circulating marine wild reservoir of VHSV capable of rapid genetic change and high evolutionary adaptation.

Such evolutionary potential for marine VHSV strains is a cause for a real concern in Scandinavia where sea-farming of rainbow trout is expanding. Shifting production from freshwater to marine aquaculture systems, fish is reared in open sea-floating net cages. While within the confines of these cages, cultured fish shares the water environment with wild marine fish, which are potential carriers of the virus and therefore the threat

of VHS outbreaks. This poses danger of host shifts from wild fish to naïve maricultured trout and the prospect of disease outbreaks in sea farms is highly possible.

It is therefore imperative that disease management/prevention strategies be developed to mitigate the impact of potential VHSV outbreaks in sea-reared rainbow trout. Such approaches include vaccine development, breeding for disease-resistant fish stocks, and immunostimulatory feed additives. A better understanding of host defenses involved is important in the development of such disease protection measures.

2.2 DNA vaccine based on rhabdovirus G protein

A DNA vaccine based on the surface glycoprotein of VHSV (Figure 3) has been shown to be successful against VHSV and confers an early cross-protective anti-viral response upon vaccination followed by specific, long-lasting protection (Kurath, 2008; Lorenzen and LaPatra, 2005; Lorenzen et al., 2002; Lorenzen et al., 1998). The complementary activities of non-specific and specific defense mechanisms most likely contribute to the high efficiency of fish rhabdovirus vaccines (Lorenzen and LaPatra, 2005), although the exact protective mechanism has yet to be clarified (Kurath, 2008; Lorenzen et al., 2002). While the early protection correlates with upregulation of the IFN-induced antiviral protein Mx, neutralizing antibodies probably contributes to the later specific immunity. However, even in the late/long-lasting protection phase, the protective effect of the vaccine does not always correlate with virus-specific antibody titers (Kurath et al., 2006; McLauchlan et al., 2003; Lorenzen et al., 1999), implying the involvement of other immune mechanisms such as cytotoxic cells (Utke et al., 2008).

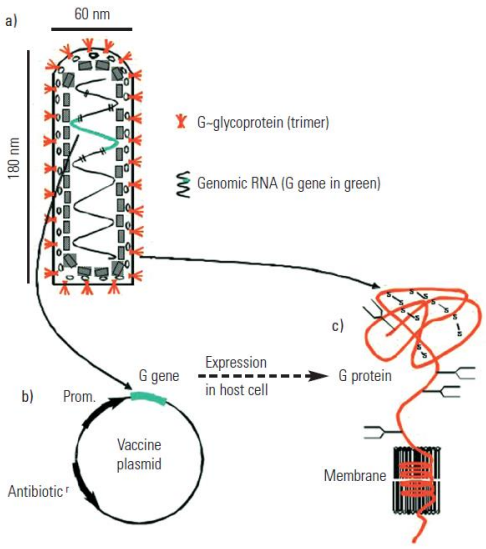

Figure 3. Schematic diagram of (A) a rhabdovirus particle, (B) the DNA vaccine, and (C) the rhabdovirus surface glycoprotein (G). The DNA vaccine is a plasmid containing the gene encoding the rhabdovirus envelope glycoprotein under the transcriptional control of a eukaryotic promoter. The G protein is a transmembrane protein found on the surface of the virus particles and on infected cell surfaces. It is stabilized by disulfide bridges (S-S) and contains oligosaccharide side chains. Following DNA vaccination, the G protein is expressed and will be present in the cell interior and on the cell surface, mimicking the natural virus infection (Lorenzen and LaPatra, 2005).

2.3 Immune response to VHSV infection and vaccination with the VHSV-G-based DNA vaccine

The fish rhabdovirus glycoprotein has been known to be a potent elicitor of the IFN response and is systemically induced following vaccination with recombinant rhabdovirus G protein or G-protein based DNA vaccine (Byon et al., 2006; Purcell et al., 2006; Byon et al., 2005; Chen et al., 2012; Verjan et al., 2008; Lorenzen et al., 2002; LaPatra et al., 2001).

Consequently, infection with fish rhabdoviruses stimulates the IFN response and induces the expression of both type I and type II IFN (IFN-γ) genes (Verrier et al., 2011; Lopez-Munoz et al., 2009; Purcell et al., 2009; Zou et al., 2007; Bernard et al., 1985), providing transcriptional data on the production of IFNs that has been demonstrated by earlier studies (de Kinkelin et al., 1982; de Kinkelin and Le Berre, 1974; de Kinkelin and Dorson, 1973). IFN-like activity was earlier reported in the sera of infected fish and inhibited virus replication (de Kinkelin et al., 1982; de Kinkelin and Le Berre, 1974; de Kinkelin and Dorson, 1973). IFN-containing supernatants and recombinant type I IFNs have also been shown to prevent fish rhabdovirus replication (reviewed by Purcell et al., 2012), Whereas type II IFNs show variable activities against rhabdoviruses (Zou et al., 2007), the protective effect of IFN-γ has been demonstrated, as well as its ability to induce antiviral responses, expression of antiviral IFN-stimulated genes (ISGs) and pro-inflammatory cytokines, and increase the expression of type I IFNs (reviewed by Purcell et al., 2012).

Infection with rhabdoviruses or G-protein-based vaccination elicits the expression of a "core" set of conserved IFN-stimulated genes (ISGs) that are typically highly induced in viral infections, as well as novel antiviral genes (Verrier et al., 2011; Byon et al., 2006; Purcell et al., 2006; Byon et al., 2005; reviewed by Purcell et al., 2012). One such ISG is the type 1 IFN-inducible myxovirus resistance (*Mx*) gene, which encodes a non-specific antiviral protein in vertebrates including teleost fishes (Verhelst et al., 2013; Jin et al., 1999; Trobridge and Leong, 1995), and is upregulated in fish injected with rhabdovirus G-expressing plasmids (Kim et al., 2000; Boudinot et al., 1998). In teleost fishes, the expression of *Mx* is stimulated by IFN-α (a type I IFN) (Berg et al., 2009; Robertsen et al., 2003) and by IFN-γ (Sun et al., 2011). The transcriptional response

kinetics post-vaccination correlates with the early nonspecific protection from lethal rhabdovirus infection (Lorenzen et al., 2002, LaPatra et al., 2001). Therefore, the antiviral activities of the IFNs, Mx, and other ISGs can partly account for the short-lived non-specific protective mechanisms (Lorenzen et al., 2002; Lorenzen et al., 2000).

In rhabdovirus infected fish, a similar response that correlates with virus titers is observed, but IFN and ISG induction is not associated with protection (Peñaranda et al., 2009; Purcell et al., 2009; Purcell et al., 2004), which could be due to the fast replication rate of fish rhabdoviruses enabling them to outpace/overtake the distribution of innate immune effectors (Purcell et al., 2012).

Specific and long-lasting adaptive immunity against fish rhabdoviruses requires the crucial involvement of infection- or vaccination-induced neutralizing and protective antibodies (Lorenzen and LaPatra, 1999). These antibodies target the rhabdovirus G protein (Lorenzen et al., 1999, 1990) and numerous studies have repeatedly demonstrated the protective effect of these antibodies (Kurath et al., 2006; Garver et al., 2005; McLauchlan et al., 2003; Corbeil et al., 2000; LaPatra et al., 2012; Traxler et al., 1999; Boudinot et al., 1998; Lorenzen et al., 1998). Nevertheless, titers of virus-specific antibodies and the protective effect of the vaccine do not always correlate (Kurath et al., 2006; McLauchlan et al., 2003; Lorenzen et al., 1999), hence other immune mechanisms such as cellular responses involving T cells and NK cells most likely also play a role.

The involvement of NK cells in the immune response against rhabdovirus infection was demonstrated in which peripheral blood leukocytes (PBLs) isolated from rainbow trout infected with VHSV or from fish vaccinated with the DNA vaccine encoding the glycoprotein of VHSV exhibited cytotoxic activity against VHSV-infected MHC I-mismatched xenogeneic target cells (Utke et al., 2008; Utke et al., 2007). In contrast, PBLs from fish vaccinated with the VHSV-N DNA vaccine did not kill xenogeneic cells, suggesting that only the G protein elicits an NK cell response (Utke et al., 2008).

T lymphocyte activity against a fish rhabdovirus is suggested by T cell clonal expansion elicited by VHSV infection and DNA vaccination (Boudinot et al., 2004, 2001). The G protein appears to contain the key T cell epitopes, as suggested by the TCR-β chain complementarity-determining region 3 profiles shared between DNA vaccination and virus infection (Purcell et al., 2012). Furthermore, specific cell-mediated cytotoxicity was demonstrated in isogenic fish and class I major histocompatibility complex (MHC I) matched cell lines. Peripheral blood mononuclear cells (PBMCs) from VHSV-infected rainbow trout killed MHC I-matched VHSV-infected target cells but not VHSV-infected xenogeneic cells (Utke et al., 2007). Likewise, PBMCs from fish immunized with VHSV G- or VHSV N-based DNA vaccines exhibited cytotoxic activity against MHC I-matched VHSV-infected target cells (Utke et al., 2008).

The immune responses to fish rhabdoviruses are reviewed in detail by Purcell and co-authors (2012).

2.4 Microribunucleic acids (microRNAs; miRNAs)

MicroRNAs are a class of evolutionarily conserved, short (~22 nt in length), endogenously expressed non-coding RNAs that have come to be recognized as key regulators of gene expression. MicroRNAs control the expression of genes post-transcriptionally by negatively regulating target gene transcript levels. They do so by binding imperfectly to the 3' untranslated region (UTR) of specific mRNA targets to reduce mRNA stability through deadenylation and mRNA decay (Schier and Giraldez, 2006) or repress (block) translation (Bartel, 2004; Filipowicz et al., 2008). They also interact with the translation machinery to decrease protein synthesis (Gu and Kay, 2010).

Initially identified as a key regulator of development in the nematode *Caenorhabditis elegans* (Wightman et al, 1993; Lee et al., 1993), miRNAs have been demonstrated to play crucial regulatory roles in a broad spectrum of biological processes including cellular differentiation, proliferation, apoptosis, developmental timing (Stefani and Slack, 2008; Bushati and Cohen, 2007; Ambros, 2004; He and Hannon, 2004), metabolism (Moore, 2013) signal transduction (Zhao et al., 2013; Inui et al., 2010; Mudhasani et al., 2008), host-virus interactions (Ghosh et al., 2009; Gottwein and Cullen, 2008). Misexpression of miRNAs is linked with several pathological conditions (Mendell and Olson, 2012; Tsai and Yu, 2010; Croce 2009; Soifer et al., 2007). Likewise, natural variants of miRNA-encoding genes or target sites in mRNAs (Saunders et al., 2007) may be responsible for phenotypic differences (Gong et al., 2014; Hu et al, 2014; Wojcicka et al., 2014; Jovelin and Cutter, 2011; Clop et al, 2006). All these emphasize the crucial functions of miRNAs in normal physiological activities.

MicroRNAs are encoded in “intergenic” or “intragenic regions” in the genome and are transcribed by RNA polymerase II into 1-3 kb primary transcripts called primary miRNAs (pri-miRNAs) (Lee et al., 2004). Pri-miRNAs are 5’-capped and poly-A tailed and folds into one or more hairpin structures, each hairpin consisting of a ~32- bp imperfect stem and a terminal loop (Skalsky and Cullen, 2010). The pri-miRNAs are processed by Drosha (a type III RNase) and its co-factor, the double-stranded RNA-binding protein Pasha (DiGeorge Syndrome Critical Region 8 Protein, DGCR8), into 70-100 nt stem-loop structures bearing 2 nt 3’ overhangs called pre-miRNAs (Gregory et al., 2004; Lee et al., 2003). The pre-miRNAs are transported from the nucleus into the cytoplasm by Exportin 5 (Bohnsack et al., 2004). The pre-miRNAs are further processed by Dicer (another type III RNase) together with TRBP (Tar RNA binding protein), removing the terminal loops to generate ~22-bp dsRNAs with 2 nt 3’ overhangs (Skalsky and Cullen, 2010). One of these two strands (guide strand) becomes the mature miRNA incorporated into the RNA-induced silencing complex (RISC), a protein complex comprising of an argonaute (Ago) protein and a GW182 protein (Hammond et al., 2000). The strand with less stable base pairing at its 5’ end is selectively loaded into the RISC complex (Tomari et al., 2007). The other strand (the passenger strand or miRNA*) is usually degraded but can also regulate miRNA homeostasis or have downstream regulatory activities (Bartel, 2009; Suzuki and Miyazono, 2011). The mature miRNA guides the RISC complex to complementary sites usually located in the 3’ untranslated regions (UTRs) of target mRNAs. Matching between the miRNA seed region (nucleotides 2-7 or 8) and the target mRNA results in mRNA degradation and/or translational inhibition/repression (Bartel, 2009). Perfect complementarity between the seed sequence and the mRNA target site generally results in endonucleolytic cleavage of the mRNA by Ago, and eventually mRNA degradation

(Cullen, 2011). Imperfect or partial matching results in repression of mRNA translation, followed by retargeting of RISC-bound mRNAs to translationally inactive processing bodies (P bodies) in the cytosol (Cullen, 2011) containing exonucleases and proteins implicated in mRNA remodeling, decapping, and deadenylation (Skalsky and Cullen, 2010). The efficiency of inhibition of mRNAs with imperfectly matched target sites depends on mRNA to miRNA expression ratio (Cullen, 2011). MiRNA biogenesis is schematically shown in Figure 1.

Each miRNA can potentially target several mRNAs and one mRNA can be targeted by multiple miRNAs (Bartel, 2009; Friedman et al., 2009; Brennecke at al., 2005; Lewis et al., 2005). Computational prediction identified >45 000 miRNA target sites in the 3' UTRs of human mRNAs and over 60% of protein-coding genes were found to be conserved targets (Friedman et al., 2009).

MiRNAs control gene expression in a cell type- or tissue- and/or time-specific fashion (Bartel 2004; Lagos-Quintana et al., 2002). They may operate in an intricate web of combinatorial controls, with a single miRNA regulating multiple targets and/or one target having many miRNA-binding sites (Bushati and Cohen 2007), thus potentially significantly affecting networks of gene expression. Because miRNAs may have considerable influence on gene expression networks, expression profiles of a few or maybe even a single miRNA may reveal changes in the expression of many mRNAs.

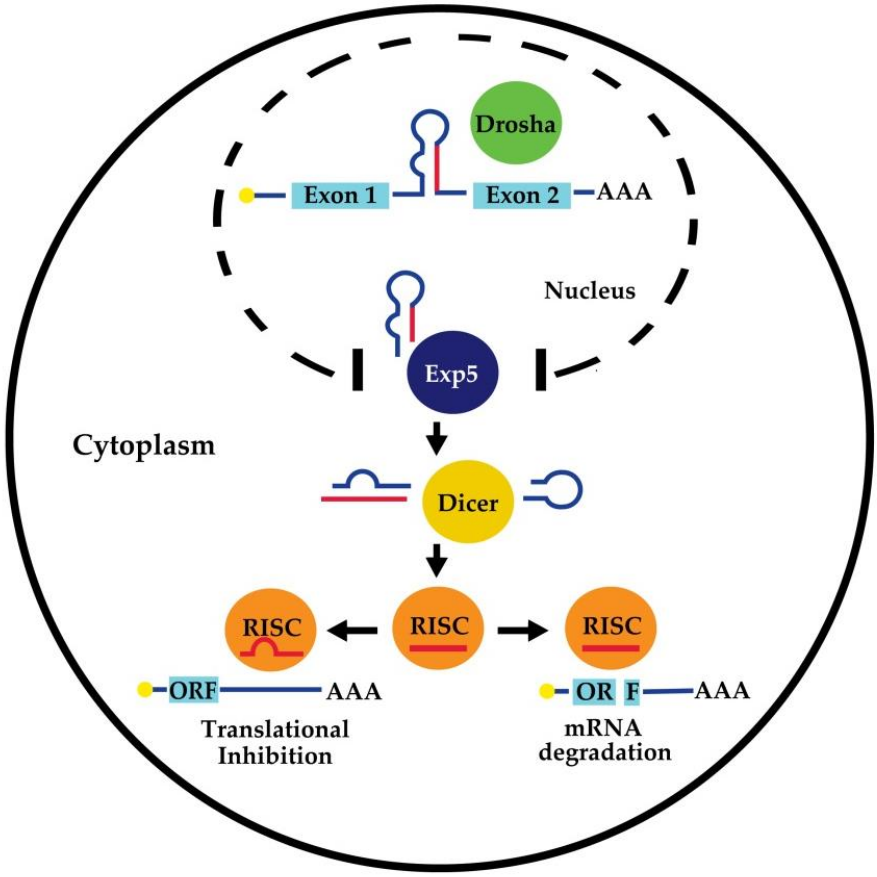

Figure 1. Biogenesis of and post-transcriptional gene silencing by miRNAs. Genes encoding miRNAs may be present in non-protein coding regions, in between protein-coding genes, or within introns. These are transcribed by RNA polymerase II (not shown) into primary miRNA transcripts (here, the miRNA is located within an intron), which Drosha processes into precursor miRNA (pre-miRNA). The pre-miRNA is subsequently exported into the cytoplasm via exportin-5. Dicer cleaves the pre-miRNA to double-stranded siRNA intermediates. The strands of the siRNA duplexes are separated during incorporation into miRNP/RISC. The mature miRNAs bound to Ago proteins guide the RISC to find cognate target mRNAs for cleavage or translational suppression depending on the level of target complementarity. (Modified from Umbach and Cullen, 2009).

Since they affect virtually all aspects of the biology of organisms, miRNAs have been studied intensively, resulting in the understanding of the aspects of the functions of miRNAs and the role of miRNA-mediated gene regulation in various physiological contexts. The breadth of the impact of these small non-coding RNAs on virtually all life processes in both normal and diseased states is so vast that they are investigated not only to elucidate cellular and organismal functions but are also being explored in the diagnosis and treatment of human diseases (Esau and Monia, 2007). The potential use of miRNAs as diagnostic and prognostic markers is being actively explored (Schwarzenbach et al., 2014; Wu et al., 2014; Zhu et al., 2014; Devaux et al., 2013; Liu et al., 2013; Madhavan et al., 2013), serving as bases for microRNA-based disease diagnostics and therapeutics. Results of studies on specific miRNAs have allowed the discovery of miRNAs with therapeutic value that have reached clinical trials (Mirna Therapeutics Press Release, 2013; Janssen et al., 2013; Lanford et al., 2010). Currently, the most clinically advanced miRNA-based therapeutic has been developed to treat Hepatitis C virus (HCV) infections (Janssen et al., 2013). The cellular liver-specific miRNA miR-122 has been known to be used by HCV to promote its own replication (Jopling et al., 2005). Miravirsen is a locked nucleic acid (LNA)-modified DNA phosphorothioate antisense oligonucleotide that antagonizes miR-122 (Janssen et al., 2013). The latest phase 2a clinical trial has demonstrated a dose-dependent efficacy of Miravirsen in reducing HCV RNA levels in patients with chronic HCV infection and the absence of signs of viral resistance and adverse effects (Janssen et al., 2013).

The first miRNA to be identified was a small RNA encoded by the *lin-4* gene in *C. elegans* (Lee et al., 1993). Since then and the initial identification of gene silencing by RNA interference (RNAi) (Fire et al., 1998), thousands of genes encoding miRNAs

have been identified in plant (Reinhart et al., 2002), animal (Ambros 2004), and viral (Cullen 2006; Kincaid and Sullivan, 2012) genomes. Thus far, over 30 000 miRNAs have been identified and annotated in 206 species, which are deposited in miRBase (miRBase Release 20; http://www.mirbase.org/) (Kozomara and Griffiths-Jones, 2011). miRBase is a comprehensive online database of miRNA sequences and annotations across a wide range of organisms and is an authority on miRNA terminology and nomenclature. Formerly called microRNA Registry (Griffiths-Jones, 2004), it is a repository of miRNA sequences, both of pre-miRNAs and those of their derivative mature miRNAs. It also provides links to online resources for miRNA target prediction and a web interface to RNA sequencing data stored in the public functional genomics data repository, Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/geo/) (Barrett et al., 2013; Edgar et al., 2002). The latest version (Release 20) of miRBase contains 30, 424 mature miRNA sequences derived from (24, 521) hairpin precursors representing 206 species. Evidence for many of these miRNAs comes from cloning and sequencing data, whereas others have been identified based on sequence similarity/homology to verified sequences from other organisms already listed in miRBase.

2.5 Cellular microRNAs in vertebrate animal host-virus interactions

Both the animal host and the virus encode miRNAs that participate as key players in the complex web of host-virus interactions. Viral miRNAs have so far been shown to play crucial roles in the establishment of long-term infection such as regulating the shift to the lytic state, host immune response evasion, and extending the life of infected cells. Viral miRNAs are also postulated to act as cellular miRNA mimics that could target host mRNAs (Kincaid and Sullivan, 2013). This section focuses on animal cellular

miRNAs and the roles that have been ascribed to them thus far. For virus-encoded miRNAs, the reader is referred to recent reviews (Kincaid and Sullivan, 2013; Grundhoff and Sullivan, 2011).

Some virus infections have been shown to alter the expression pattern of cellular miRNAs (Fu et al., 2014; Zhang et al., 2014; Hicks et al., 2013; Tambyah et al., 2013; Buggele et al. 2012; Cameron et al., 2008; Pepini et al., 2010; Houzet et al., 2008; Varnholt et al., 2008), revealing an essential function for miRNAs in the interactions of the hosts with viruses.

Cellular miRNAs have been shown to positively or negatively impact virus infection. For instance, miR-122 has been shown to stabilize hepatitis C virus (HCV) genome, protect it from exonuclease degradation, enhance its replication and to stimulate protein synthesis (Conrad and Niepmann, 2013; Li et al., 2013; Wilson and Huys, 2013; Jopling et al., 2005). Epstein-Barr virus (EBV) and enterovirus induce specific host miRNAs which enable them to replicate (in culture) by targeting mRNAs of proteins which may function in anti-viral defense (Ho et al., 2011; Linnstaedt et al., 2010).

On the other hand, several studies have reported that mammalian cellular miRNAs directly target virus sequences and inhibit their replication (Zheng et al., 2013; Huang et al., 2007; Otsuka et al., 2007; Pedersen et al., 2007; Lecellier et al., 2005; see also review by Russo and Potenza, 2011). For example, mouse miR-24 and miR-93 target *Vesicular stomatitis virus* genes encoding the large (L) protein and phosphoprotein (P) (Otsuka et al., 2007). A number of IFN-β-induced miRNAs in human cells have been

shown to target *Hepatitis C virus* (HCV) sequences and inhibit HCV replication in vitro, thus contributing towards the antiviral properties of IFN-β (Pedersen et al., 2007). Other mammalian miRNAs regulate the expression of host genes that either directly or indirectly negatively impact virus infection (Gao et al., 2013; Zhang et al., 2013; Terrier et al., 2013; Smith et al., 2012; Wang et al., 2009; Triboulet et al., 2007). Such cellular miRNAs may potentially be used as antiviral effectors in mammalian cells (discussed in review Manuscript 3).

The importance of cellular miRNAs as antiviral effectors in mammalian cells is suggested by observations that miRNA production is impaired in Drosha- or Dicer-deficient mice and cultured cells, which boosted viral replication and increased susceptibility to infection (Otsuka et al., 2007; Triboulet et al., 2007). Furthermore, some mammalian viruses have the ability to suppress RNAi mechanisms. For instance, influenza A virus has been shown to target Dicer (Matskevich and Moelling, 2007), whereas HIV-1 has been reported to actively suppress the expression of a cellular miRNA that regulates the expression of a host gene whose protein product benefits HIV-1 (Triboulet et al., 2007).

It is believed that since cellular miRNAs are evolutionarily conserved (Bartel, 2009), it is probable that viruses would evolve ways to circumvent inhibition by endogenous cellular miRNAs, although how they do this is currently not understood. Cullen (2013) proposed a number of possible mechanisms that viruses might employ in order for viruses to evade inhibition by cellular miRNAs. These include 1) blocking miRNA function; 2) mutating in the 3' UTR targets complementary to cellular miRNAs; 3) evolving very short 3' UTRs which may allow translating ribosomes to remove mRNA-

bound RISC; and 4) having structured (with “high level” of secondary structures) long UTRs which restrict RISC recruitment (Cullen, 2013).

2.6 MicroRNAs in immune responses

Dicer and the cellular RNAi machinery have been shown to influence the proliferation, survival, lineage choice, and cytokine production during T cell differentiation (Muljo et al., 2005). Mice with conditional Cre-mediated *dicer 1* deletion had reduced T cell numbers in peripheral lymphoid organs. CD4+ T cells had defective miRNA processing and showed impaired development and abnormal cytokine production (Muljo et al., 2005).

A number of microRNAs are known to modulate various features of mammalian immunity, ranging from immune cell development and differentiation, hematopoiesis, proliferation, cell fate establishment and maintenance, Toll-like receptor (TLR) signaling and cytokine production, antigen presentation, antibody switching, and T cell receptor signaling (Baltimore et al., 2008; Lindsay, 2008; Sonkoly et al., 2008), all of which contribute to infectious disease outcomes, as well as to the advance of immune-relevant disorders.

Differential expression of specific miRNAs influence immune cell phenotypes. For instance, B lymphocytes in mouse bone marrow selectively upregulate miR-181 and miR-223, but not undifferentiated progenitor cells, which reflects their role in regulating lineage differentiation (Chen et al., 2004). Peripheral blood mononuclear cells and T lymphocytes in humans afflicted by autoimmune and inflammatory diseases likewise differentially express particular miRNAs (Liu et al., 2012; Stagakis et al.,

2011; Li et al., 2010; Pan et al., 2010; Pauley et al., 2008; Stanczyk et al., 2008). In B cell malignancies, miR-155 has been shown to be present in high levels in B lymphocytes (Costinean et al., 2006; Eis et al., 2005; Kluiver et al., 2005; Metzler et al., 2004; reviewed by Lindsay, 2008). All these indicate the crucial role of miRNAs in shaping and maintaining the proper functions of immune cells and the development of immunological disorders when miRNA expression becomes abnormal.

Other studies have shown that activation of innate immune signaling alters the expression of specific miRNAs, which modulate acute inflammatory responses by controlling the production of inflammatory cytokines or targeting innate immune signaling proteins (reviewed by Lindsay, 2008). For example, exposure to IL-1β and TNF-α or stimulation of TLR-2, -4, and -5, upregulate miR-146a in macrophages and alveolar/bronchial epithelium, which consequently negatively regulates innate immune responses (Perry et al., 2008; Taganov et al., 2006). Likewise, innate immune response activation induces the expression of miR-155, which suppresses the release of inflammatory mediators (reviewed by Lindsay, 2008).

Whereas specific miRNAs exert activities in particular contexts, a single miRNA, miR-155, plays versatile roles in regulating numerous immune functions, both innate and adaptive. It has been shown to regulate members of the tumor-necrosis factor family receptors and their ligands (Tili et al., 2007; Costinean et al., 2006). Apart from its involvement in B cell lymphomas as mentioned earlier, it has been shown to be critical for normal T cell and B cell differentiation and antibody production since knockout mice show impaired B and T cell function (Rodriguez et al., 2007; Thai et al., 2007; Vigorito et al., 2007). The activity of miR-155 is needed to produce

immunoglobulin class-switched plasma cells (Vigorito et al., 2007). By modulating cytokine production, miR-155 regulates T helper cell differentiation and germinal center reaction to promote the development of an optimal T cell-dependent antibody response (Thai et al., 2007). Its role in DC function is suggested by observations that miR-155-deficient DCs are inefficient antigen presenters (Rodriguez et al., 2006). It controls differentiation of helper T cells into its various subsets (O'Connell et al., 2010; Vigorito et al., 2007; Rodriguez et al., 2006) and influences of regulatory T cell development (Kohlhaas et al., 2009). It is highly upregulated in activated mouse cytotoxic T cells (CTLs) and in antigen-specific effector CTLs (Gracias et al., 2013) and was essential for optimum CTL responses against pathogens and for generating pathogen-specific CTL memory (Dudda et al., 2013; Gracias et al., 2013). It has also been shown to play a role in CTL proliferation and in regulating CTL responsiveness to type I IFN (Gracias et al., 2013). It has also been shown to positively regulate IFN-γ production in human NK cells (Trotta et al., 2012). Furthermore, miR-155 overexpression has been observed in virus-infected cells, in which it suppresses apoptosis and promotes proliferation of HCV-infected hepatocytes (Zhang et al., 2012) and reduces NF-κB signaling, contributes to immortalization of EBV-infected B cells, and inhibits innate immune response to latent EBV infection (Lu et al., 2008). Therefore, a tight regulation of miR-155 expression is vital in order to ensure normal immune cell functions and prevent disease development. This also makes miR-155 as a good therapeutic target in numerous immune-related and infectious diseases.

2.7 MicroRNAs in teleost fish

Genes encoding miRNAs have been identified in teleost fish genomes (*in silico* prediction based on sequence homology) (Yang and He, 2014; Barozai, 2012; Loh et al., 2011), as well as conserved 7-mer sequences in the 3' UTR of teleost fish genes identical to recognized miRNA-binding sites (Andreassen et al., 2009). MiRNAs have been demonstrated as crucial regulators of gene expression during in zebrafish development, indicating the presence of functional RNAi pathway that operates in teleost fish as it does in other organisms (Giraldez et al., 2006). The use of zebrafish as a model to study the role miRNAs play during vertebrate development has contributed significantly towards revealing some common themes in vertebrate miRNA functions (Takacs and Giraldez, 2010).

With about 23, 000 teleost fish species (http://fishbase.org/home.htm Accessed 10 April 2014), teleost fishes comprise almost half of the extant vertebrate lineage. Teleosts exhibit astonishing variability in different aspects of biology from genomes to ecology and inhabit diverse aquatic environments of the world (Nelson, 2006). Therefore, they are good subjects for research on various biological aspects especially those pertaining to evolution.

Despite the enormous species diversity and the economic and cultural importance of fish in both wild fisheries and aquaculture, teleost fish miRNAs are currently underrepresented in the miRNA repository miRBase (http://mirbase.org), being limited to 8 species (miRBase release 20, accessed 25 April 2014). Among others, these include miRNAs identified in the model species zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) and the aquaculture fish species common carp (*Cyprinus carpio*) and Channel

catfish (*Ictalurus punctatus*). A total number of 1250 teleost fish pre-miRNA hairpin sequences and 1044 mature miRNAs are currently listed in mirBase (http://mirbase.org, accessed 25 April 2014) and are summarized in Table 1. Zebrafish miRNAs are currently the most numerous teleost miRNAs in miRbase.

Table 1. MicroRNA entries for teleost fish species held in Release 20 of miRBase (accessed 25 April 2014).

Fish species	Common name	Sequences available in miRbase	Reference(s)
Oryzias latipes	Medaka	168 precursors, 146 mature	Li et al., 2010; Tani et al., 2010
Fugu rubripes	Fugu	129 precursors, 108 mature	miRBase (communicated by) Mihaela Zavolan
Tetraodon nigroviridis	Pufferfish	132 precursors, 109 mature	miRBase (communicated by) M. Zavolan
Danio rerio	Zebrafish	346 precursors, 255 mature	Chen et al., 2005; Kloosterman et al., 2006 + many others
Cyprinus carpio	Common carp	134 precursors, 146 mature	Zhu et al., 2012; Yan et al., 2012
Paralichthys olivaceus	Japanese flounder	20 precursors, 38 mature	Fu et al., 2011
Hippoglossus hippoglossus	Atlantic halibut	40 precursors, 37 mature	Bizuayehu et al., 2012
Ictalurus punctatus	Channel catfish	281 precursors, 205 mature	Xu et al., 2013

The usefulness of zebrafish as a model to investigate miRNA functions in vertebrate embryogenesis allowed the elucidation of the mechanisms by which target mRNAs are regulated (Takacs and Giraldez, 2010; Morton et al., 2008; Giraldez et al., 2006; Schier and Giraldez, 2006;). Accordingly, many of these teleost miRNAs have been identified in studies that analyzed miRNAs profiles in different zebrafish developmental stages and in selected zebrafish cell lines (Wei et al., 2012; Chen et al., 2005), as well as in

general sequencing activities (Soares, 2009; Kloosterman et al., 2006). Indeed, many of the studies undertaken on teleost fish miRNAs focused on developmental profiling. Apart from zebrafish, miRNA developmental profiles have also been carried out on another model fish species medaka (Tani et al., 2012), and in the aquaculture fish Atlantic halibut (Bizuayehu et al., 2012).

MiRNAs from a few other fish species which await inclusion in miRbase are also available in literature, which include those identified from sequencing experiments and those predicted *in silico* (Table 2). While many of these miRNAs are evolutionarily conserved and are thus homologous to previously identified miRNAs from other organisms including other fishes, unique new miRNAs have been characterized (e.g. in Atlantic salmon) (Andreassen et al., 2013; Bekaert et al., 2013).

In functional terms, teleost fish miRNAs are less characterized compared with their mammalian homologues. Functional investigation has lagged behind the increasing reports on miRNA discovery and profiling in different biological settings. Apart from the activities of zebrafish miRNAs in different aspects of embryonic development (Tacaks and Giraldez, 2010), involvement of teleost fish miRNAs has been investigated in various physiological contexts in different species such as muscle growth and development (Yan et al., 2013), vitellogenesis (Cohen and Smith, 2013), oogenesis (Juanchich et al., 2013), aging (Terzibasi-Tozzini et al., 2014), liver-specific metabolism (Mennigen et al., 2014a,b), osmotic regulation and osmotic stress response (Yan et al., 2012a,b), organ regeneration (Yin et al., 2012; Thatcher et al., 2008; Yin et al., 2008), and immune responses to bacterial infection (Ordas et al., 2013; Wu et al., 2012; Xia et al., 2011).

Table 2. Teleost fish species in which microRNAs have been identified other than those included in miRBase.

Fish species	Common name	Reference(s)
Gadus morhua	Atlantic cod	Johansen et al., 2011
Ictalurus punctatus	Channel catfish	Barozai, 2012
Lates calcarifer	Asian seabass	Xia et al., 2011
Megalobrama amblycephala	Blunt snout bream	Yi et al., 2013
Nothobranchius furzeri	Turquoise killifish	Terzibasi-Tozzini et al., 2014
Oncorhynchus mykiss	Rainbow trout	Mennigen et al., 2014a,b; Juanchich et al., 2013; Mennigen et al., 2013; Trattner and Vestergren, 2013; Yang and He, 2014; Ma et al., 2012; Salem et al., 2010; Ramachandra et al., 2008
Oreochromis niloticus	Nile tilapia	Xiao et al., 2014; Tang et al., 2013; Yan et al. 2013; Huang et al., 2012; Yan et al., 2012a,b,c
Paralichthys olivaceus	Japanese flounder	Fu et al., 2011
Salmo salar	Atlantic salmon	Andreassen et al., 2013; Bekaert et al., 2013; Barozai, 2012; Reyes et al., 2012
Hypophthalmichthys nobilis	Bighead carp	Chi et al., 2011
Hypophthalmichthys molitrix	Silver carp	Chi et al., 2011
Various cichlid species	Lake Malawi cichlids	Loh et al., 2011

Of the teleost miRNAs, those from zebrafish have the most functional data and validated targets (Takacs and Giraldez, 2010). In tilapia, miRNAs with experimentally validated targets have also been reported (Yan et al., 2013; Yan et al., 2012a,b). These include miR-206, which targets insulin-like growth factor 1 (IGF-1) mRNA to regulate growth in tilapia (Yan et al., 2013); miR-30c, which modulates the expression of heat shock protein 70 (HSP70) and consequently regulates responses to osmotic stress in the kidney (Yan et al., 2012); and miR-429, which silences the osmotic stress transcription factor 1 (OSTF1) and affects osmosensory signal transduction in the gill epithelium (Yan et al., 2012).

Having a fully sequenced genome and being a model for functional genomics research, computationally predicted targets for zebrafish miRNAs are also available and can be obtained from TargetScanFish (http://www.targetscan.org/fish_62/) (Ulitsky et al., 2012). The role of the majority of teleost fish miRNAs and their targets is currently unknown.

The miRNAs identified and characterized from other teleost species (Table 2), pending their inclusion in miRBase, will greatly expand the number of annotated teleost fish miRNAs which would be a valuable resource for functional genomic studies and future research (explore evolution of fishes and vertebrates in general), especially studies dealing with the role of miRNA-mediated gene control in the expression of economically important traits. The lack of functional data for many of the teleost fish miRNAs currently offers a vast opportunity for scientific investigation. Functional studies will shed light on whether the many of the roles of these teleost miRNAs are conserved among teleosts and their mammalian homologues. MiRNAs most likely influence the expression of numerous mRNAs in different cell and tissue types in diverse biological contexts in fish, as do many miRNAs whose functions have been elucidated in other organisms.

2.8 Do homologous miRNAs perform conserved functions across species?

It is interesting to look into the functional conservation of homologous miRNAs across the vertebrate lineage. The high degree of conservation of miRNAs among distantly related vertebrate species implies involvement in generic biological processes and functional conservation. Accordingly, miRNAs are identified by numbers in a continuous numbering system across all living species. A three-letter prefix is used to

indicate the species in which the individual miRNA was found. Functional conservation across multiple species may be valuable when working with model organisms to unravel miRNA functions in various aspects of vertebrate biology.

For example, miR-146a and miR-146b are highly conserved between humans and teleost fishes. In mammals, the expression of these two members of the miR-146 family is stimulated by bacterial lipopolysaccharide (LPS) and the inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) (Taganov et al., 2006) and by bacterial (Nahid et al., 2011) and viral (Ho et al., 2014) infections. They act in the negative feedback regulation of TLR signalling by targeting the TLR signalling intermediates IL-1 receptor-associated kinase (IRAK) and TNF receptor-associated factor 6 (TRAF6) (Boldin et al., 2011; Curtale et al., 2010; Bhaumik et al., 2008; Taganov et al., 2006). Mir-146a has also been shown to target the transcription factors IFN regulatory factor 5 (IRF-5) and sugnal transducer and activator of transcription 1 (STAT-1), thus inhibits IFN signalling (Tang et al., 2009). Because innate immune signalling pathways are evolutionarily conserved among the vertebrates (van der Vaart, et al., 2012; Aoki et al., 2008; Purcell et al., 2006), and the teleost fish orthologues of IRAK and TRAF6 (Phelan et al., 2005) also possess miR-146 putative target sites (Ordas et al., 2013), the functional conservation of miR-146 feedback control of TLR signalling between mammals and teleost fish may be rationally anticipated. In zebrafish, miR-146a/b orthologues have been shown to be upregulated following bacterial infection (Ordas et al., 2013).

Likewise, the liver-specific miR-122 is conserved among the vertebrates and participates in modulating numerous hepatic processes including the metabolism of fat and cholesterol in mammals (Hsu et al., 2012; Hu et al., 2012; Tsai et al., 2012). A study in rainbow trout indicated that miR-122 may have a conserved function in postprandial lipogenesis in the liver in mammals and in fish and may have evolved in vertebrates to play a role in liver-specific metabolic activities (Mennigen et al., 2014).

However, while the induction of miR-146a/b in bacteria-infected zebrafish embryos suggests an association with innate immune responses, knockdown of miR-146a/b did not strongly affect the induction of teleost *irak1*, *irf5*, *stat1*, and *traf6* transcripts (Ordas et al., 2013). Whereas protein levels of IRAK1, IRF5, STAT-1, and TRAF6 were not checked and miR-146a/b may function by translational suppression (Ordas et al., 2013), it is also possible that miR-146a/b have different mRNA targets between fish and mammals.

Likewise, miRNAs may show quantitative variation in function and different target mRNA repertiore among vertebrate species, as indicated for miR-122 (Mennigen et al., 2014).

Recent reports also demonstrated that differences in miRNA expression pattern and hence variation in regulation and function, increases with larger physiological differences among vertebrate species (Ason et al., 2006) Consequently, evolvability of miRNA target sites between fish and humans is higher compared with that between chimpanzee and human (Xu et al., 2014). Thus, despite the highly conserved sequences, homologous miRNAs may evolve specialized functions in different species. This

implies that evolution of RNAi takes place at the 3' UTR miRNA recognition region of the regulated mRNAs rather than in the miRNA seed region. This may reflect some unique features of their physiology arising from the need to diversify physiological strategies to adapt to the various lifestyles and environments.

2.9 General discussion of the project

The fundamental role of miRNAs in virtually all biological activities and the numerous gaps in the current knowledge of their exact roles in these various processes in organisms necessitate continued scientific research on the involvement of miRNAs in every biological problem of interest. In this thesis, the potential role of miRNAs in rhabdovirus infection in rainbow trout and in fish responses to vaccination with a DNA vaccine based on the fish rhabdovirus glycoprotein was investigated.

Although virus infection in mammals and bacterial infection in teleost fish have been shown to modulate the expression of cellular miRNAs, studies on miRNA expression profiling following virus infection and/or vacination in teleost fish are presently limited. Identification of IFN-induced miRNAs in response to both VHSV infection and VHSV-G-based DNA vaccination reflects the ability of the virus and the glycoprotein to stimulate fish innate immune responses, particularly IFN and IFN-induced mechanisms.

As reported in Manuscript I, the induction of the clustered miRNAs miR-462 and miR-731 by IFNs can be accounted for by an IFN-stimulated response element (ISRE) and gamma IFN activation site (GAS) in the promoter sequences upstream of the miR-462/731 locus. It was also found that the teleost miR-462/731 cluster is an orthologue

of an ancestral miR-191/425 cluster in vertebrates, which is known to be involved in cell cycle control in humans. Functional specialization between the teleost miR-462/731 and human miR-191/425 clusters is a consequence of a change in detailed genome position and mode of regulation, presumably along with evolution of the 3' UTR miRNA recognition sites in the mRNA. Accordingly, *in silico* analysis indicates very dissimilar target profiles between fish and humans, which reflects evolution of diverse species-specific functions of orthologous miRNAs among vertebrates.

In manuscript II, the IFN-dependent expression of miR-462/731 was confirmed and was shown to be induced also by the natural infection-mimicking VHSV-G-based DNA vaccine, demonstrating a common miRNA response to both infection and vaccination. These results further demonstrated that the two miRNAs may be involved in IFN-mediated antiviral activities, acting as antiviral effectors thereby contributing to the early protective effect of the DNA vaccine.

Finally, manuscript III (review) provides a state-of-the-art update on the roles of miRNAs in antiviral defense in vertebrates.

The findings in this thesis add up to the knowledge on the functional characterization of miRNAs in the context of host-virus interactions in teleost fishes and should stimulate interest among scientists to pursue research in this new and interesting area of fish biology where potential research opportunities abound and is relatively underexplored. The two IFN-induced miRNAs identified in the project may be novel components of the antiviral response in teleost fishes. Their potential use as biomarkers of virus infection or vaccine-induced immunity should be explored along with the

search for genetic variability related to disease immunity. They may also be potential targets for therapeutic intervention against viral diseases in aquaculture.

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MANUSCRIPT 1.

Two virus-induced microRNAs known only from teleost fishes are orthologues of microRNAs involved in cell cycle control in humans

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Short title: Regulation and evolution of two vertebrate microRNAs in Rainbow trout

Significance: MicroRNAs (miRNAs) are important regulators of gene expression involved in virtually all biological processes. Two clustered miRNAs, miR-462 and miR-731, identified thus far only in teleost fishes, lack functional characterization. Here, we found a strong induction of miR-462/731 in virus-infected fish, correlating with interferon sensitive promoter elements. The miR-462 and miR-731 were shown to be orthologues of the ancestral miRNAs, miR-191 and miR-425, respectively, which in humans are involved in cell cycle control. The findings demonstrate that some miRNAs despite high conservation have evolved into specialized functions in different vertebrate lineages. This is the first report on the presence of interferon-inducible miRNAs in teleost fish. We speculate that RNA interference might play an important role in innate antiviral immunity in fish.

Abstract: MicroRNAs (miRNAs) are ~22 base-pair long non coding RNAs which regulate gene expression in the cytoplasm of eukaryotic cells by binding to specific target regions in mRNAs thereby mediating transcriptional blocking or mRNA cleavage. Of the more than 1000 human miRNAs which have been described so far, more than 50% are conserved across vertebrate species. Through their fundamental roles in cellular pathways, gene regulation mediated by miRNAs has been shown to be involved in many physiological processes such as embryonic development, toxicological response, tumour formation, and immunological reactions. The miRNAs miR-462 and miR-731 have so far only been identified in teleost fishes without data on functional aspects. We here report a strong upregulation in rainbow trout following inoculation with the highly virulent rhabdovirus *viral hemorrhagic septicaemia virus*. Gene synteny analyses along with gene sequence conservation suggested that the teleost fish miRNAs have evolved from an ancestral miRNA cluster known as miR-191/425. In humans, the genes encoding these miRNAs are found in an intron of the *DALDR3* gene and functionally associated with cell cycle regulation. In contrast to this, we found the teleost miR cluster in an intergenic position with upstream IFN-related regulatory elements. Stimulation of fish cell cultures with the TLR3 ligand poly I:C accordingly induced upregulated expression of the teleost miRs 462/731 while no stimulatory effect on miRNA-191/425 expression was seen in human cell lines. Despite high sequence conservation, evolution has thus resulted in different functional roles of these orthologous miRNA clusters in different vertebrate lineages.

Keywords: MicroRNA; Regulation; Fish; Rhabdovirus; Interferon response; miR-462; miR-731; miR-191 cluster; Evolution

Introduction:

MicroRNAs are small non-coding RNA molecules (22-25 nucleotides long) that modulate gene expression in the cells by pairing with complementary mRNAs whereby they inhibit translation of genes into proteins (1). Following their first description in 1993 in invertebrates (2), it has been found that these molecules are remarkably conserved within vertebrates and as a statement of their importance, it has been estimated that more than 60% of human protein coding genes are targets of miRNAs (3). New miRNAs are continuously being discovered, identified by a continuous numbering system in miRBase (4). In humans, animals, and plants, more than 20000 unique miRNAs have been described and related to a range of different functions. The complexity of miRNA regulation is increased by the fact that each miRNA often appears to target several genes, often involved in the same pathway or the same overall phenotype, thereby reinforcing their functionality (5). In this study, we have observed a very strong induction of two clustered miRNAs, miR-462 and miR-731 (herein referred to as the miR-462/731 cluster), in the liver of a teleost fish, the rainbow trout (*Oncorhynchus mykiss*), following infection with the fish rhabdovirus *viral hemorrhagic septicaemia virus* (VHSV). Analysis of regulatory sequences surrounding the miR-462/731 locus revealed interferon (IFN) inducible and other immune-related promoter elements, suggesting involvement of the miRs in anti-viral immune responses. By gene synteny and sequence homology analysis, we found that the teleost miR-462 and -731 are orthologues of miR-191 and -425, respectively, found in mammals as well as in the ancestral elephant shark belonging to cartilaginous fishes. Despite high sequence conservation of miRs across vertebrate species, data on the functional role of miRs in terms of which genes they target and how they are regulated have appeared to be less consistent and dependent the physiological specialization of the individual

vertebrate lineages (6, 7). Here we describe how these two teleost miRNAs differ from their human orthologues in terms of genome position and regulation and discuss how these differences may reflect functional specialization.

Results:

Identification of regulated miRNAs in the liver of VHSV infected fish

Microarray-based examination of miRNA regulation in the liver tissues from VHSV infected as compared to non-infected control fish using 1492 unique miRNA specific probes detected 120 miRNAs expressed significantly above the background level determined as a mean of all probe signals. Of these, 13 showed more than two-fold up regulation in infected compared to control fish (Fig. 1A) and among these, miR-462 and -731 were particularly upregulated (>50-fold). The strong expression levels of the two miRNAs were confirmed by qPCR, showing that miR-462 was upregulated ~20-fold and miR-731 ~15-fold (Fig. 1B).

Identification and characterization of an immune induced polymerase II specific promoter upstream miR-462 and 731 in fish

We found miRNAs miR-462 and miR-731 particularly interesting as both were highly regulated in the liver of VHSV-infected fish. miR-462 and miR-731 have been described so far only in teleost fish and the mirBase currently lists only miR-462 and miR-731 as identified in zebrafish (*Danio rerio*) (8, 9), medaka (*Oryzias latipes*) (10) and channel catfish (*Ictalurus punctatus*) (where only miR-462 has been identified so far; (11).

A search in Ensembl database (http://www.ensembl.org/index.html) further showed that these miRNAs are found as an intergenic miRNA cluster between the housekeeping genes inosine monophosphate dehydrogenase 2 (*IMPDH2*) and DALR anticodon binding domain containing 3 (*DALRD3*) on chromosome 8 in the zebrafish genome and that the two miRNAs comprising the miR-462 cluster are separated by 125 nucleotides.

Available genomes of fish and higher vertebrates were searched for the *IMPDH2* and the *DALRD3* genes and we found evolutionary variants of the two miRNAs in the region of these genes for the various genomes. In the teleost fish genomes the mature miR-462 and -731 were found to be fully conserved in both seed sequence and genome location. We aligned the upstream sequence from *IMPDH2* to the miRNA cluster and the downstream cluster until *DALRD3* using the BLAST bl2seq option at NCBI Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and found some sequence motifs, which were conserved in teleost fish. We used the conserved sequence motifs together with conserved sequences in the premature miRNAs to sequence a 1000 bp long stretch of the rainbow trout genome flanking the 5´-side of the cluster from the last part of the miR-731 hairpin gene (Fig. 2). In order to look for indications of a promoter upstream of the miRNA cluster, we searched this sequence using the Transcription Element Search System (TESS; http://www.cbil.upenn.edu/tess) and BIOBASE immune specific binding sites prediction (http://www.*biobase*.de). In this way, we identified an interferon (IFN)-stimulated response element (ISRE), G/A/T + GAAANNGAAA + G/C + A/T/C, (12-15) 828 bp upstream the start of the mature miR-462 (Fig. 2), indicative of type I interferon inducibility. This motif is 100% conserved among teleost fishes, indicating conserved regulation (Fig. S1). Another motif, the purine box 1 (PU.1: GAGGAAGT) involved in the development of granulocytes, macrophages, and lymphocytes in jawed vertebrates (16-17) was conserved among teleosts (Fig. S1). In the rainbow trout sequence we further identified the TATA element (18), indicative of RNA polymerase II-dependent expression and a motif which was repeated twice and resembled the gamma interferon activated site (GAS) element (IFN-gamma activated sequence; TTN CNN NAA; (19-21). In addition, a sequence from the rainbow trout

GGTTTTTTC was found to be conserved in the other fish as well, but we were not able to identify this as any known promoter motif (Fig. 2).

Identification of miR-462 and miR-731 orthologues in mammalian genomes

Synteny analysis between the zebrafish and human genomes showed the two intergenic/non-intronic miRNAs, miR-191 and miR-425, within the region flanked by *DALRD3* and *IMPDH2* instead of miR-462 and miR-731 present in teleost fish genomes (http://www.ensembl.org/index.html) (Fig.3 A-B). Alignment of miR-191 and miR-425 with miR-462 and miR-731, respectively, showed high homology especially in the antisense strand and a full conservation in the seed area (Fig.3 C). Furthermore, by aligning the fish and the human *DALRD3* sequences, we found that the miR-191/425 cluster sequences were absent in the fish *DALRD3* locus (data not shown). Together, these findings suggest that the intragenic teleost fish miR-462/731 cluster is phylogenetically related to the miR-191/425 cluster. Interestingly, while comparing several mammalian genomes from the Ensembl database, we noted that whereas the human, macaque, and mamorset miR-191 cluster is present intragenically in an intron of *DALRD3* (Fig 3 A-B), this is not the case for all mammalian genomes.

Stimulation of miRNA expression in cell culture

Stimulation of rainbow trout liver cells (RTL-W1) with the TLR3 ligand poly I:C upregulated the expression of the miR-462/731 cluster (Fig. 4A&B). On the other hand, infection of RTL-W1 cells with VHSV did not stimulate an upregulation of the miRNAs but rather a tendency was seen for downregulation (Fig. S3). The presumed miRNA promoter sequence was checked for its ability to promote the expression of the green fluorescent protein (GFP) in rainbow trout macrophage cell line (RTS-11) transfected with a GFP reporter plasmid construct carrying the suspected miRNA

promoter sequence upstream the GFP gene. Flow cytometry analysis confirmed that these cells could be stimulated by poly I:C to produce GFP, while the IFN-γ-stimulating ligand phytohemagglutinin (PHA) failed to induce GFP expression (Fig. 4C-G).

Subsequent stimulation of HeLa cells with poly I:C induced the expression of the IFN-induced gene *ISG12* but not miR-191 and miR-425 (Fig. S4). We used HEK293T cells as control as these are known not to be poly I:C inducible due to lack of TLR3 expression (22, 23) and showed neither *ISG12* regulation nor significant regulation of the examined miRNAs.

Phylogenetic analysis

Based on the high homology of the seed regions in the non-teleost miRNA-191/425 and teleost miRNA-462/731 clusters (Fig. 3C), conserved clustering pattern, related genomic location as well as lack of genomic co-existence, we hypothesized these miRNA clusters to represent evolutionary orthologues. We therefore performed phylogenetic analysis based on the mature sequences of the miR-191 and miR-462 cluster miRNAs retrieved from miRbase to elucidate their evolutionary history in vertebrates.

Unrooted neighbor-joining (NJ) bootstrapped distance trees (1000 replicates) for both miR-191 and miR-462 cluster miRNAs showed that the miRNAs of teleosts and those of a cartilaginous fish (elephant shark; *Callorhinchus milii*) clustered within distinct clades (Fig. 5A-B). The teleost clade is separated with large genetic distances from those of the cartilaginous fish and higher vertebrates, with the largest genetic distance from the latter. On the other hand, miRNAs of diverse higher vertebrates all clustered together and were thus almost identical.

Discussion:

The data presented here are the first functional and evolutionary analyses of a cluster of two virus induced microRNAs (miR-462/731) only found in teleost fishes. MicroRNAs are endogenous small RNA molecules which have appeared to play an important role in posttranscriptional regulation of protein expression in many living organisms (1, 24). Their high level of conservation across vertebrate species suggests involvement in generic biological processes, but this is not consistently supported by currently available data (6, 7). We report here that the miR-462/731 cluster in teleost fishes is orthologous to the miR-191/425 cluster present in other vertebrates, but differ in gene regulation and detailed genomic position. The results suggest that the miRNA clusters has evolved different functional roles in different vertebrate lineages.

Based on teleosts belonging to an evolutionary early vertebrate lineage, we initially thought that the miR-462/731 cluster represented an ancestral form of the mammalian miR-191/425 cluster. However, the recently discovered presence of the miR-191 cluster in the cartilaginous fish *C. milii*, which has the slowest evolving genome of all known vertebrates (25), suggested that the miR-191 cluster has existed in the common ancestor of cartilaginous fishes and bony vertebrates. The probability of convergent evolution of two miRNAs is considered to be small (26-27) and animal miRNAs generally exhibit a low rate of secondary loss (28). Together with their consistent position relative to the *DALRD3* locus as well as the association of the two miRNAs comprising the given miRNA cluster in all genomes analyzed, this further supports the genetic relation of the miR-462 and miR-191 clusters.

Phylogenetic analysis accordingly demonstrated that the teleost fish miRNA cluster represented a distinct subclade within their non-teleost homologues. This basically

suggests that the fish miRs 462 and 731 should be considered as homologues of the non-teleost miRs 191 and 425, respectively. The large genetic distance that according to the phylogenetic analysis (Fig. 5) separates the teleost fish miRNA cluster from those of other vertebrates indicates that teleost miRNAs in relative terms have undergone accelerated changes but is based on a very high conservation in the seed region among the non-teleosts in combination with a few changes in the teleost nucleotide sequence outside of the seed region. The most important evolutionary adaptation within the teleost fish thus appeared to be the local regulatory elements outside the miRNA cluster.

All available teleost fish genomes have the miR462 and miR-731 positioned in a region between the *DALRD3* and *IMPDH2* loci whereas humans and some other mammals have the homologous miR-191 cluster in an intron in the *DALRD3* gene. The intergenic position of the miR-191cluster in the genome of the primitive cartilaginous fish, *C. milii* (25) suggests that a translocation of the miR-191 locus into the *DALDR3* intron has occurred during vertebrate evolution in the primate lineage including human, macaque, and marmoset.

Our search for regulatory elements upstream the teleost miR-462 cluster supported the involvement of the miRNAs in innate immune functions. Presence of ISRE and GAS elements suggested that their expression can be enhanced by the binding of IFN-induced transcription factors while the TATA-box indicated that the expression of miR-462 and miR-731 is steered by a polymerase type II promoter as reported for other miRNAs (29). We found an ISRE element, which itself shows a remarkable conservation among vertebrates (15) and is a binding site for IFN-regulatory factors (IRFs) and IFN-stimulated gene factor 3 (ISGF3). Upregulation of the miR-462/731

cluster in fish RTL-W1 cells stimulated with the TLR3 ligand poly I:C supported that the expression of the miRNAs was related to IFN induction (Fig. 4A&B). The observed downregulation of miR-462/731 cluster in VHSV-infected RTL-W1 cells (Fig. S3) was contradictory to what was observed in the fish, but correlated with a lack of IFN induction in the RTL-W1 cells following VHSV infection and could thus reflect that VHSV is able to suppress IFN induction (30) in this cell line.

The presence of the GAS element indicates IFN-γ-regulated transcription. However, attempts to induce miR-462/miR-731 expression by stimulation of RTS-11 cells with PHA or LPS gave only weak signal in immunofluorescence microscopy (not shown) and could not be shown by flow cytometry (Fig. 4G). These cells have been shown to upregulate levels of IFN-γ-inducible protein 10 (IP-10), as well as the key IFN-γ intracellular signalling molecule STAT 1 in response to PHA stimulation (31) and our findings might thus reflect that the cloned promoter region is incomplete in terms of IFN-γ sensitive motifs. Like the ISRE and GAS motifs, the leukocyte differentiation related transcription factor (PU.1) binding sequence also appeared to be a conserved immune-related regulatory element in teleost genomes (16-17; 32-33) upstream of the miR-462 cluster. In rainbow trout, PU.1 is known to be upregulated tissue specifically in the muscle, liver, intestine, brain, head kidney, and in macrophages in primary cell culture following LPS stimulation (32).

While IFN induced expression of other miRNAs as well as the involvement of miRNAs in antiviral immune mechanisms has been described earlier (34-35), this is to our knowledge the first report describing the involvement of the teleost miR-462/731 cluster in response to virus infection. In contrast, the expression of the human miR-191 cluster has not been directly associated with immune responses. Accordingly, we were

not able to induce miR-191 nor miR-425 in poly I:C-stimulated human cell lines HeLa and HEK-293T despite the high up-regulation in HeLa cells of the IFN-stimulated gene *ISG-12*. In addition, the miR-191/425 cluster was not among the many inducible miRNAs found in a virus replicon-bearing human hepatoma cell line, nor were they upregulated by the addition of ribavirin and/or IFN-β to these cells (36). The regulation of the intragenic miR-191 cluster in humans appears to be different, possibly controlled by various sequence elements such as estrogen response elements (37-38), among other regulatory elements (39-40), and by epigenetic mechanisms (41). Furthermore, the implication of the human miR-191 cluster in various physiological processes (42-46) and in a number of pathologies and human neoplasias (37-41; 47-48) indicate a wide and diverse expression pattern, target repertoire, and regulatory roles in diverse processes in multiple organs and tissues. As discussed by Ason at al. (7), differences in miRNA expression profiles – and thereby differences in regulation – between vertebrate species increases with increased physiological differences. Accordingly, Xu et al. (6) found a very high evolvability of miRNA target sites between fish and humans and low evolvability between chimpanzee and human using cross-linking immunoprecipitation data.

In conclusion, our study showed that the IFN-regulated miR-462/731 cluster in teleost fishes is an orthologue of the ancestral vertebrate miR-191/425 cluster (present in a wide range of non-teleost vertebrates from primitive cartilaginous fish to humans) but that the teleost variant has evolved differently with respect to functional roles. In contrast to the miR-462/731 cluster in teleost genomes, the human miR-191/425 cluster does not appear to be involved in immunological functions. Thus, the regulatory changes through evolution of the orthologous miRNA clusters have resulted in their functional specialization/diversification in different vertebrate lineages. This was

supported by our *in silico* target prediction analysis suggesting very different target profiles in fish and humans (Tables S2-S5) but requires further functional analysis as well as 3´UTR target sequence validation. Our findings support earlier analyses showing that miRNA sequence conservation among distantly related species may not necessarily imply functional conservation and that higher miRNA expression variation reflects functional diversity associated with larger differences in physiology (7). The evolution of IFN-related response elements associated with the miR-462 cluster in teleost fishes seems to reflect some unique features of the innate immune response in teleost fishes. Specialization in the function of these miRNAs across this highly diverse vertebrate group could have resulted from the need to diversify innate immune defence strategies as an adaptation to the aquatic environments with varying temperatures and frequent exposure to water borne viruses. Further functional studies are expected to reveal how the teleost miR-462 and -731 are involved in the response to virus infection.

Materials and methods:

Total RNA purification from liver of diseased trout

Total RNA from the liver was isolated and purified using the miRNeasy Mini kit (Qiagen cat.no. 217004), quantified, and quality tested by using the nanodrop system (Thermo Scientific, USA) measuring RNA at OD 260 nm and impurities by the OD 260nm / OD 280 nm ratio followed by stabilization in RNA stable (Biomatrica, USA) according to the manufacturers protocol. A description of the viral challenge experiment is presented in supplementary Text S1.

Expression analysis of miRNAs and ISG-12 gene

qPCR for miRNAs was performed using the sequences of the mature dre-miR-462, dre-miR-731, and hsa-miR-191 as forward primers together with the universal primer from the kit used to prepare cDNA (Text S1). The mature miRNA sequences can be found in the miRBase at http://www.mirbase.org/ (49). For detection of human *ISG12* mRNA transcripts, human *ISG12* sequence was used as forward primer together with the universal primer from the kit, as above.

Real-Time PCR reactions were run on a Mx3000P machine (Agilent Technologies, CA, USA) in reaction volumes of 25 µl containing a master mix solution (Brilliant® SYBR® Green QPCR Master Mix cat. no. 600548, Agilent Technologies, CA, USA), specific primers, ROX, water, and 5 µl of cDNA template (prepared as described in Text S1) diluted x100. The program was set to 10 min at 95°C, 40 cycles of 20 sec at 94°C and 1 min at 60°C with collection of fluorescent data. Melting curves were determined by denaturing PCR products for 1 min at 95˚C followed by a ramp down to 55˚C for 30 sec followed by a gradual 0.2˚C/sec climb to 95˚C continuously recording

fluorescence. Transcript levels were measured as Ct values and normalized to trout *snoRNA-U23, miR-16, let-7a,* or *snoRNA U6* for miRNAs and to the housekeeping gene *18S rRNA* for human *ISG-12* mRNA. PCR Primers for both miRNA and mRNA detection are listed in Table S1. Values presented are means of normalized Ct values from 3 cell culture wells + SD. The sizes of all PCR products were verified by inspection of the dissociation curve and by gel electrophoresis.

Genomic localisation of the miR-462 cluster and upstream region in sequenced fishes

The gene cluster containing miR-462 and miR-731 was localised on the chromosome 8 of the zebrafish genome in Ensembl release 48 (50). Synteny analysis was used to find homologous miRNA genes in all teleost fish genome assemblies present in Ensembl as well as in selected genomes of higher vertebrates including human. This was done by searching these genomes for the genes *IMPDH2* and *DALRD3* flanking the miR-462 cluster in the zebrafish chromosome 8. The miRNAs were localized using the mature miRNA sequences and the NCBI 2seq blast algorithm. Upstream of these miRNAs, immunological relevant promoter motifs were found by the use of the on-line database search tools Transcription Element search System (TESS; http://www.cbil.upenn.edu/tess) and BIOBASE (www.biobase.de) as well as blast (bl2seq) (http://www.ncbi.nlm.nih.gov/genbank/) alignments for previously published motifs from fish. All searches were performed on the 1000 nt upstream of the miR-462 locus. Selected vertebrate miR-191 and miR-425 sequences were aligned with the zebrafish miR-462 and miR-731, respectively, in the CLC main Workbench (CLC, Denmark) multiple alignment using default settings.

Cloning and sequencing of the premature miR-462 cluster and its 5'- flanking region:

Two PCR amplicons generated (Text S1) were cloned into the TOPO TA Cloning Kit vector ® (Invitrogen). Plasmids were transformed in DH5α cells and cultured in LB medium with ampicillin (100 μg/ml) and screened for inserts by T7-M13 primers as specified by Invitrogen. Plasmids of positive clones were purified using the Plasmid DNA Purification kit (Qiagen). Inserts were verified by gel electrophoresis of 5' *EcoRI* and 3' *XbaI* double digests. Confirmed clones were sequenced commercially (DNA Technology, Denmark) using the cloning primers.

Construction of a plasmid containing the 5'-flanking region and a reporter gene EGFP

The 5'-flanking region of the miRNA cluster was amplified using a forward primer composed on the 5' side by an inserted *restriction site* for *Bgl*II (underlined) followed by the sequence of the ISRE element containing region roughly 1000 nt upstream the miRNA cluster, 5'-GGAGATCTAGAAGTGAAAGTGAAATACA-3' *in combination with a reverse primer* flanked on its 5' side by a *Xba*I *restriction site (underlined)*, 5'-CGCTCTAGA TACCCGCTAACACCACTACTGAGT-3'. For the amplification of the presumed promoter area the following conditions were used: 1 cycle of 94°C for 3 min, 14 cycles of 94°C for 60 sec, 54°C decrease 0.5ºC per cycle for 60 sec and 68°C for 2 min, 19 cycles of 94°C for 60 sec, 46°C for 60 sec and 68°C for 1 min, with a terminal step of 5 min at 68°C. The PCR product (838 bp) was digested by *Bgl*II and *Xba*I after gel purification and cloned into pcDNA3.1/CT-GFP (Invitrogen) in which the CMV promoter had been excised by *Bgl*II and *Xba*I digestion. The presence of the presumed ISRE promoter in the pcDNA3.1/CT-ISREprom-GFP plasmid was confirmed by

restriction enzymes and by bidirectional sequencing (DNA Technology, Denmark). Concentrations of plasmids were determined by Nanodrop spectrophotometry (Thermo Fisher Scientific, Germany).

Cell culture transfection and ligand stimulation studies

For flow cytometry studies of promoter activation, 10^6 RTS-11cells (51) were electroporated with 2µg pcDNA3.1/CT-ISREprom-GFP plasmid using the Microporator MP-100 system (Invitrogen, USA) for 24 well plates and 10µl tips using two pulses of 1300 volts at a pulse width of 20. Immediately following electroporation, either 100µg/ml poly I:C (Sigma-Aldrich GmbH, Steinheim, Germany; CAS # 42424-50-0) formulated 1:2 in DOTAP as previously described (57) or 20 µg/ml phytohaemagglutinin (PHA) was added directly to the medium. As controls, mock cells or cells transfected with plasmids without the presumed ISRE promoter or plasmid without EGFP were used. Following 48 hrs of stimulation at 15^0C, cells were trypsinized and detached from plates, stained with CELL LAB ApoScreen™ Propidium Iodide (PI) (Beckman Coulter, USA) and run in a Beckman Coulter FC-500 flow cytometer. Graphs were prepared in the Kaluza software (Beckman Coulter, USA) gating out PI stained dead cells. Rainbow trout RTL-W1 cells (52) and human HeLa and HEK293T cells stimulated with poly I:C or inoculated with VHS virus (fish cells only) were examined for miRNA and gene expression as described above.

Bioinformatics analysis

Mature miRNA sequences of miR-191/425 and miR-462/731 were retrieved from miRbase. Elephant shark miR-191/425 stem-loop sequences were accessed from NCBI Nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore/). Multiple sequence

alignment was carried out using Clustal Omega through the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) server (http://www.ebi.ac.uk/Tools/msa/clustalo/) (53). The aligned sequences were then analysed by Neighbour-Joining method using Jukes-Cantor nucleotide distance measure and 1000 replicates using CLC Genomic Workbench 7.0.3. The trees were subsequently visualised as unrooted neighbor joining (NJ) trees. Putative targets of miR-191 and miR-462 were predicted with the TargetScanHuman Release 6.2 algorithm, which predicts target mRNAs in vertebrate genomes (http://www.targetscan.org/vert_61/) (3). Determination of the potential targets of teleost fish miR-462 and miR-731 was performed using the TargetScanFish Release 6.2 algorithm, which predicts target mRNA in the zebrafish genome (www.targetscan.org/fish_62/) (54).

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Figures

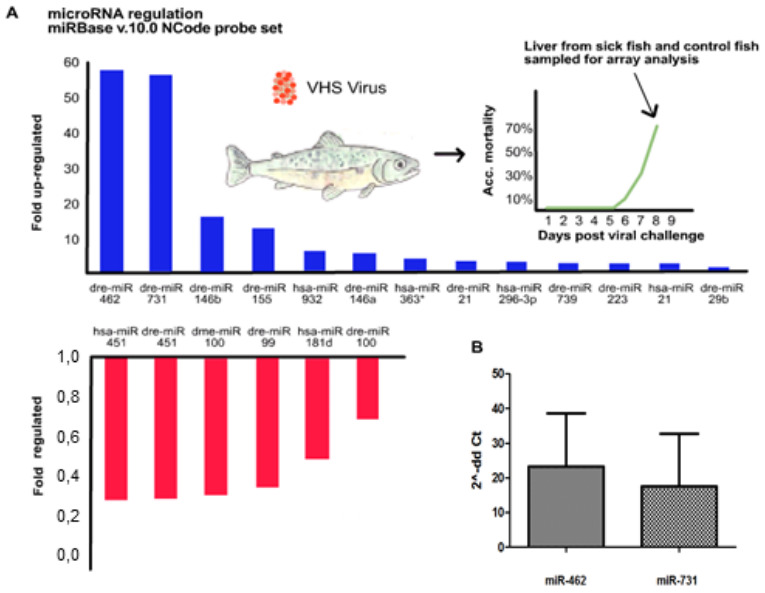

Fig. 1: miR-462 and miR-731 are highly up-regulated in the liver of rainbow trout infected with *viral hemorrhagic septicaemia virus* (VHSV). (A) The data presented are from a microarray experiment where the Ncode V2 probeset covering miRBase v.9 was used to detect up-(blue) and down-regulated (red) miRNAs in liver samples from diseased rainbow participating in an infection trial (shown in insert). Fold regulation was calculated by dividing the mean intensity of arrays with RNA from diseased fish with the mean of arrays with RNA from control fish. Up-regulated miRNAs are shown as blue bars and down regulated miRNAs as red bars. Probes are denoted according to convention and as in miRBase. Organisms: dre = *Danio rerio* , hsa = *Homo sapiens* and dme = *Drosophila melanogaster*. (B) qPCR validation of miR-462 and miR-731 expression in the liver of VHSV-infected rainbow trout. Fold regulation was calculated from the mean values of duplicate measurements using the $\Delta\Delta C_t$ method, where Relative Expression = $2^{-\Delta\Delta C}{}_t$ and $\Delta\Delta C^t = (C_t \text{ infected} - C_t \text{ reference gene}) - (C_t \text{ non-infected} - C_t \text{ reference gene})$. The reference gene used for normalization was omy-snoRNA U23. miRNA expression was analyzed in 6 VHSV-challenged fish relative to 6 unchallenged fish.

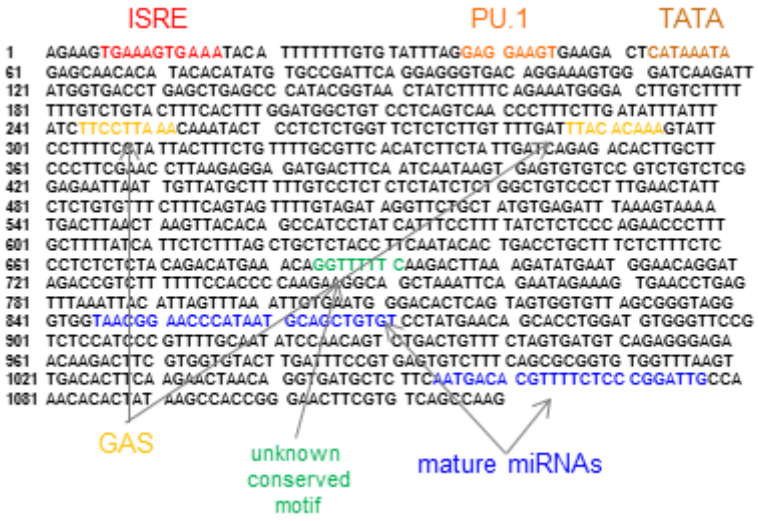

Fig. 2: Potential miRNA promoter area in rainbow trout. The conserved motif sequences ISRE and PU.1 found upstream of the two miRNAs (Fig. S1) together with the miRNA sequences allowed design of primers for sequencing of the area from the ISRE element to the end of the mature miR-731. Several motifs known from immunologically relevant promoters were identified in this sequence. Element key colors: Red: ISRE element of fish according to Castro et al. 2008 (15); Orange: The PU.1 element (17); Brown: The TATA-box; Yellow: consensus GAS elements (15); Blue: mature miRNAs up-regulated during VHSV infection; Green: unknown conserved sequence. Based on these motifs the upstream sequence is presumed to be an IFNγ regulated class 2 polymerase promoter. The identified sequence is part of the intergenic area between the genes dalrd3 and impdh2 in the trout genome and comprises 1119 bp. The location between dalrd3 and impdh2 was also conserved in the other fish.

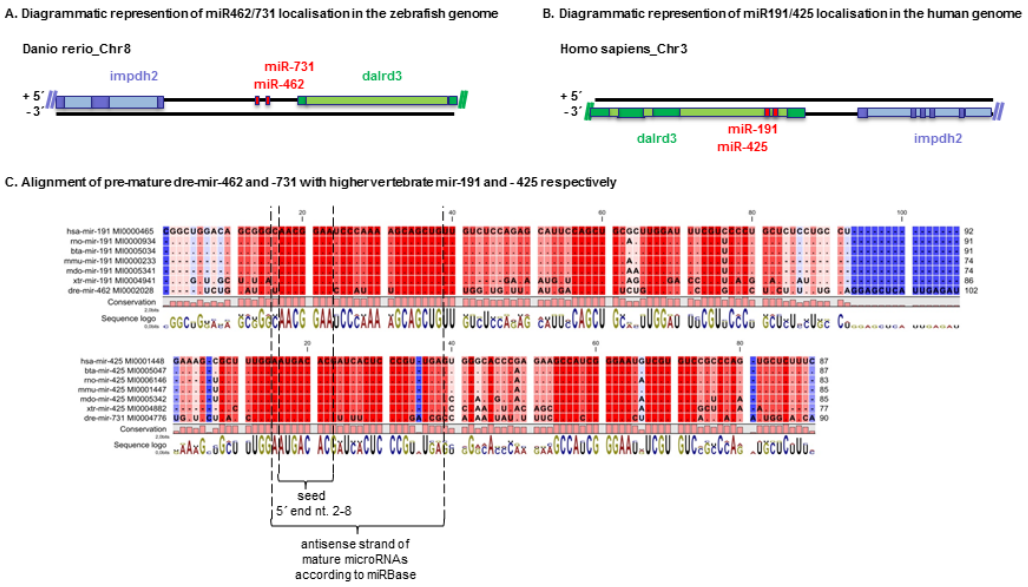

Fig. 3: miR-462 and miR-731 in fish are orthologous of miR191 and miR-425 in higher vertebrates. (A-B) Diagram of the location of the miR-462731 cluster in zebrafish and the miR-191/425 cluster in humans. (C) Alignment of pre-miRNA sequences from various vertebrate species. Highlighted below the alignments is the location of the mature antisense miRNA. hsa = *Homo sapiens* (human), bta = *Bos taurus* (cow), rno = *Rattus norvegius* (rat), mmu = *Mus musculus* (mouse), mdo = *Monodelphis domestica* (opossum), xtr = *Xenopus tropicalis* (frog), and dre = *Danio rerio* (Zebrafish). See Figure S2 for an alignment including more species.

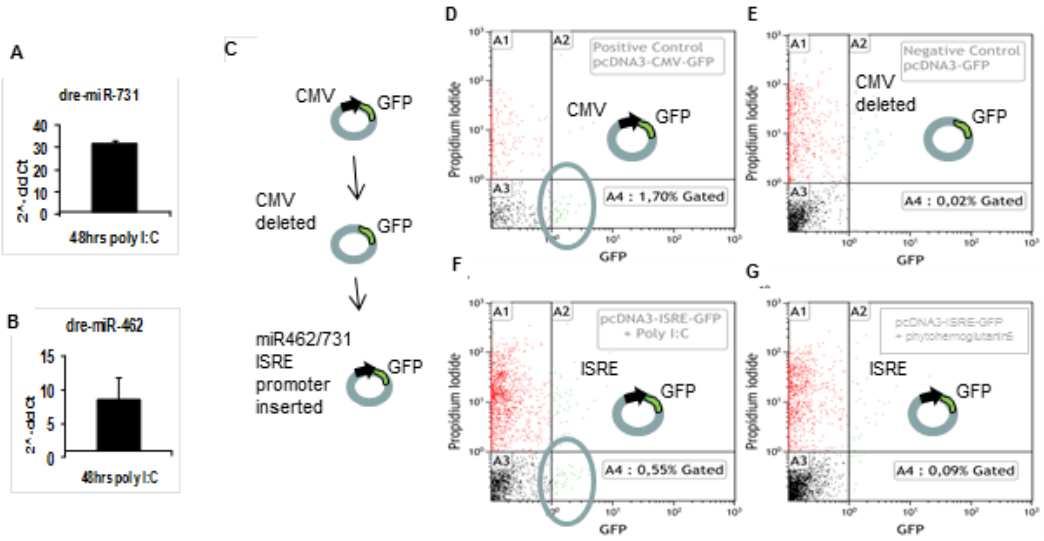

Fig. 4: The promoter steering expression of miR462 and miR731 can be activated by poly I:C. (A-B) Trout liver cells RTL-W1 in culture up-regulated miR-731 and -462 following stimulation with poly I:C. (C-G) Using various plasmid constructs transfected into rainbow trout RTL-W1 cells followed by stimulation with poly I:C, some cells showed higher levels of green fluorescence compared to controls and non-transfected cells indicating activation of the suspected miRNA promoter by poly I:C. Blue circles in flow cytometry diagrams indicate cells with green fluorescence higher than the gated negative cells. Cells transfected with a positive control plasmid containing the CMV promoter followed by the GFP reporter and a negative control plasmid containing only the GFP reporter without any promoter were used to gate for GFP positive cells (quadrant A4) and GFP negative cells (quadrant A3) respectively. Red dots indicate dead cells according to their staining above background with propidium iodide (quadrant A1 and A2). Such cells were not considered in the analysis. Using the same experiment we were not able to show up regulation in cells following stimulation with the TLR-4 ligand phytohemoglutanin (I).

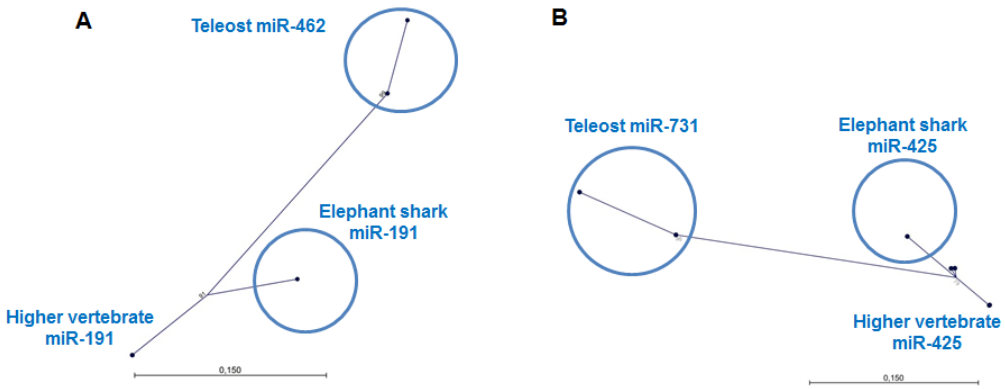

Fig. 5: Phylogenetic analysis of (A) miR-191 and miR-462 and (B) miR-425 and miR-731. Unrooted neighbor-joining (NJ) trees with bootstrap values from 1000 replicate analysis indicated at the nodes as percentage values. In 4B, high similarity among the sequences originating from higher vertebrates makes the end nodes appear overlapping, thereby mimicking a single node.

Supplementary Information

Table S1. Primers used in qPCR experiments.

Gene name*	Accession number	Primer sequence (5'→ 3')[a]
dre-miR-462	MIMAT0001855	TAACGGAACCCATAATGCAGCT
dre-miR-731	MIMAT0003761	AATGACACGTTTTCTCCCGGATCG
omy-snoRNA U23	AJ009730	GCCCATGTCTGCTGTGAAACAAT
hsa-miR-191	MIMAT0000440	CAACGGAATCCCAAAAGCAGCTG
hsa-miR-425	MIMAT0003393	AATGACACGATCACTCCCGTTGA
hsa-miR-16	MIMAT0000069	TAGCAGCACGTAAATATTGGCG
hsa-let-7a	MIMAT0000062	TGAGGTAGTAGGTTGTATAGTT
hsa-ISG-12	X67325.1	CAAATTCTGCATCTCCAGAGG
hsa-18S rRNA	K03432.1	TTCCGTAGGTGAACCTGCGGA

*dre = Danio rerio (zebrafish); hsa = Homo sapiens (human); omy = *Oncorhynchus mykiss* (rainbow trout)

[a] All primers are forward primers used together with a universal reverse primer supplied in the QuantiMir RT Kit

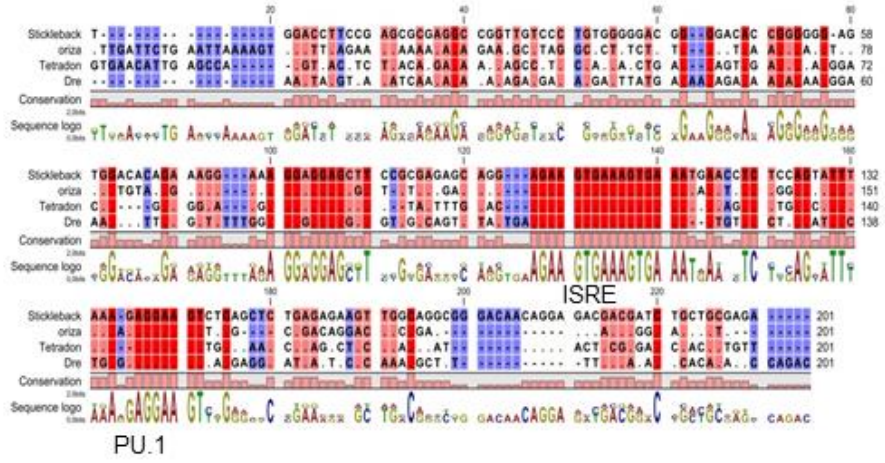

Figure S1. Conservation of specific sequence motifs upstream miR-462 and miR-731. The area upstream of the two miRNAs (-1000nt) were retrieved from selected fish genomes in the UCSC database. By alignment of these sequences conserved motifs were found which were later identified as ISRE and PU.1.

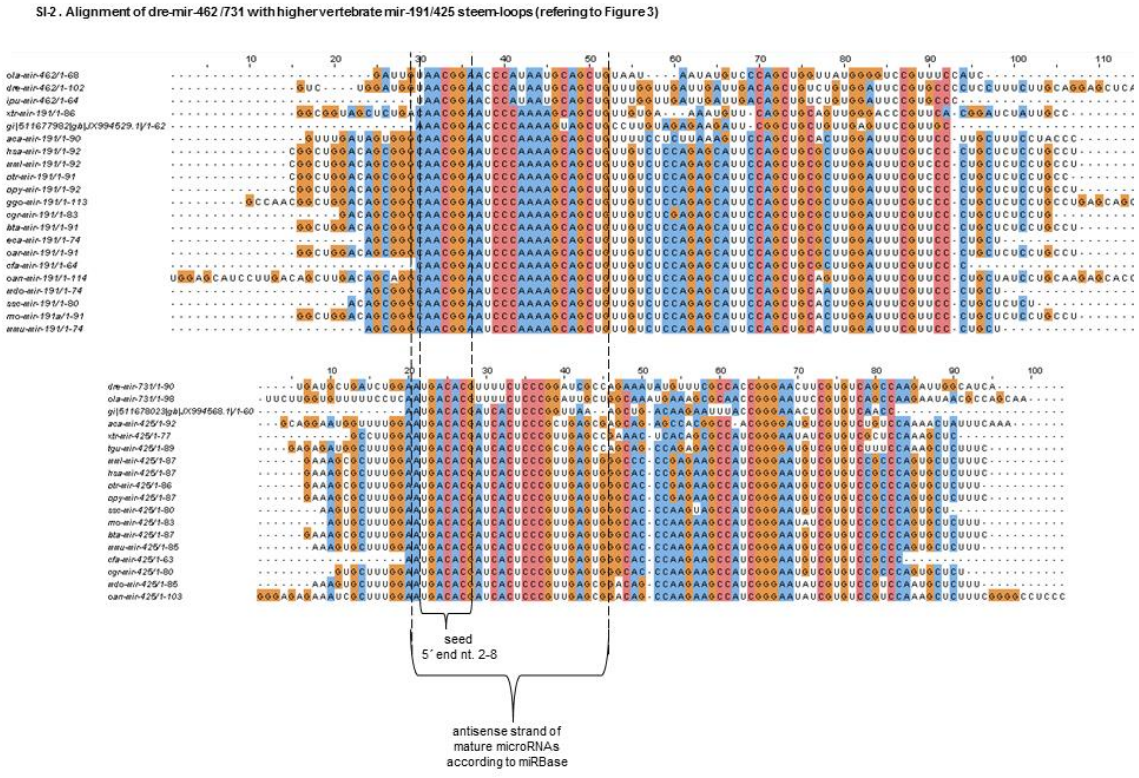

Figure S2. Alignment of dre-miR462/731 with higher vertebrate miR-191/425 stem loops (referring to Figure 3).

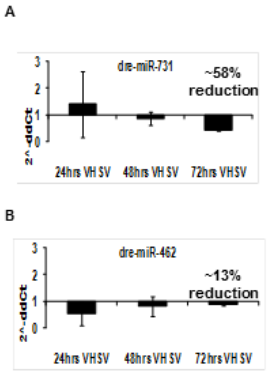

Figure S3. Rainbow trout liver cells RTL-W1 in culture did not up-regulate miR-731 and -462 24, 48 and 72 hrs. following infection with VHS virus.

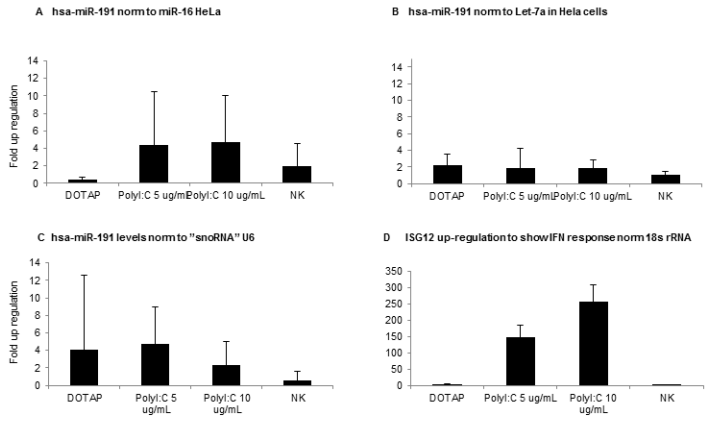

Figure S4. Human miR-191 and -425 regulation is not steered by an poly I:C activated promoter. (A-C) Immune stimulation by poly I:C does not induce up regulation of neither miR-191 in human Hela cells. Normalization to miR-16 (A) showed a tendency towards regulation following polyI:C stimulation, but it was not significant. Normalizations of the same data to other commonly used normalization genes Let-7a (B) and snoRNA U6 (C) did also not show any significant changes. Cells were either mock treated with DOTAP or treated with DOTAP formulated poly I:C at 5 or 10 ug/ml concentrations. The negative control (NK) refers to untreated cells to which the other group-data were normalised. Standard deviations are shown. (D) Despite that there was no significant up-regulation of hsa-miR191 following polyI:C stimulation it was verified that the polyI:C treatment did indeed induce a strong concentration dependent interferon response, as shown by up regulation of ISG12, in the Hela cells. Note that the values on the y-axis in (D) are much higher than in (A-C). The samples were also checked for the regulation of miR-425 by PCR which also showed no significant regulation (data not shown). Human embryo kidney cells HEK293t cells were used as negative control cells because an innate cellular response in these cells cannot be induced by poly I:C. Accordingly these did neither regulate ISG12 nor any of the miRNAs (data not shown).

Table S2. Selected putative targets of miR-191 in vertebrate genomes predicted using the TargetScan Release 6.2 algorithm and ranked by their probability of conserved targeting (P_{CT}). (Only top-ranked targets are shown.)

Target Gene	Representative transcript	Gene name	Vertebrates whose miR-191have predicted targets in human mRNA orthologues									
			Rat	Frog	Cow	Opossum	Rhesus	Dog	Chimpanzee	Mouse	Platypus	Horse
BDNF	**NM_001143805**	brain-derived neurotrophic factor	+	+	+	+	+	+	+	+	+	+
CASK	**NM_001126054**	calcium/calmodulin-dependent serine protein kinase (MAGUK family)	+	+	+	+	+	+	+	+	+	+
TMOD2	**NM_001142885**	tropomodulin 2 (neuronal)	+	-	-	+	+	+	+	+	-	+
IFFO2	**NM_001136265**	intermediate filament family orphan 2	+	-	+	-	+	+	+	+	-	+
NEURL4	**NM_001005408**	neuralized homolog 4 (Drosophila)	+	-	+	+	+	+	+	+	-	-
ZNF362	**NM_152493**	zinc finger protein 362	-	-	+	+	+	+	+	-	+	+
CDK6	**NM_001145306**	cyclin-dependent kinase 6	-	-	+	-	+	+	+	+	-	+
MAPRE3	**NM_012326**	microtubule-associated protein, RP/EB family, member 3	+	-	+	+	-	+	-	+	+	+
RCC2	**NM_001136204**	regulator of chromosome condensation 2	+	-	-	+	+	+	+	-	-	+
AMMECR1	**NM_001025580**	Alport syndrome, mental retardation, midface hypoplasia and elliptocytosis chromosomal region gene 1	+	+	+	+	+	+	+	+	+	+
MSI1	**NM_002442**	musashi homolog 1 (Drosophila)	-	-	+	-	+	+	+	-	-	+
NFIA	**NM_001134673**	nuclear factor I/A	+	+	+	+	-	+	+	+	+	+
WIZ	**NM_021241**	widely interspaced zinc finger motifs	+	-	-	-	+	+	+	+	-	+

SLC12A5	**NM_001134771**	solute carrier family 12 (potassium/chloride transporter), member 5	-	-	-	-	+	+	+	+	-	+
GAP43	**NM_001130064**	growth associated protein 43	+	+	+	+	+	+	+	+	+	+
AJAP1	**NM_018836**	adherens junctions associated protein 1	-	-	+	+	+	+	+	+	+	+
FZD5	**NM_003468**	frizzled family receptor 5	+	-	+	-	+	+	+	+	-	-
ATP2B2	**NM_001001331**	ATPase, Ca++ transporting, plasma membrane 2	+	-	+	-	+	+	+	+	-	+
ZCCHC24	**NM_153367**	zinc finger, CCHC domain containing 24	+	-	-	-	+	+	+	+	-	+
RNF139	**NM_007218**	ring finger protein 139	+	-	+	+	+	+	+	+	-	+
FUBP3	**NM_003934**	far upstream element (FUSE) binding protein 3	-	+	+	+	+	+	+	+	+	+

Table S3. Putative targets of miR-425 in vertebrate genomes predicted using the TargetScan Release 6.2 algorithm and ranked by their probability of conserved targeting (P_{CT}).

Target Gene	Representative transcript	Gene name	Vertebrates whose miR-425 have predicted targets in human mRNA orthologues									
			Rat	**Frog**	**Cow**	**Opossum**	**Rhesus**	**Dog**	**Chimpanzee**	**Mouse**	**Platypus**	**Horse**
NUFIP2	**NM_020772**	nuclear fragile X mental retardation protein interacting protein 2	+	-	-	-	+	+	+	+	+	-
FOXJ3	**NM_001198850**	forkhead box J3	+	+	-	-	+	+	+	+	+	-
C11orf41	**NM_012194**	chromosome 11 open reading frame 41	-	-	-	-	+	-	+	-	-	-
STRN	**NM_003162**	striatin, calmodulin binding protein	-	-	-	-	+	+	+	-	-	-
ZAK	**NM_133646**	sterile alpha motif and leucine zipper containing kinase AZK	-	-	-	-	-	+	+	-	+	-
ATP5G3	**NM_001002258**	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C3 (subunit 9)	+	-	-	-	+	+	+	+	-	-
FAM135B	**NM_015912**	family with sequence similarity 135, member B	+	-	-	-	+	+	+	+	-	-
CBL	**NM_005188**	Cas-Br-M (murine) ecotropic retroviral transforming sequence	-	-	-	-	+	+	+	-	+	-
WWC3	**NM_015691**	WWC family member 3	-	-	-	-	+	+	+	-	+	-
RALGAPA2	**NM_020343**	Ral GTPase activating protein, alpha subunit 2 (catalytic)	-	-	-	-	+	-	+	-	-	-
DUSP16	**NM_030640**	dual specificity phosphatase 16	+	-	-	-	+	-	+	-	-	-

CSNK1G1	**NM_022048**	casein kinase 1, gamma 1	+	-	-	-	+	+	+	+	+	-
HLF	**NM_002126**	hepatic leukemia factor	-	-	-	-	-	+	+	-	-	-
FBXO41	**NM_001080410**	F-box protein 41	-	-	-	-	+	+	+	-	-	-
KLF3	**NM_016531**	Kruppel-like factor 3 (basic)	+	-	-	-	+	+	+	-	-	-
ANTXR1	**NM_032208**	anthrax toxin receptor 1	-	-	-	-	+	-	+	+	-	-
FAM133B	**NM_001040057**	family with sequence similarity 133, member B	-	-	-	-	+	+	+	-	-	-
IGF1	**NM_000618**	insulin-like growth factor 1 (somatomedin C)	+	-	-	-	+	-	+	+	-	-
TLK1	**NM_001136554**	tousled-like kinase 1	-	-	-	-	+	-	+	-	+	-
MPP5	**NM_022474**	membrane protein, palmitoylated 5 (MAGUK p55 subfamily member 5)	-	-	-	-	+	+	+	-	-	-
CPLX2	**NM_001008220**	complexin 2	-	-	-	-	-	+	+	-	-	-

Table S4. Putative targets of miR-462 in the zebrafish genome predicted using the TargetScanFish Release 6.2 algorithm and ranked by their probability of conserved targeting (P_{CT}).

Target gene	Representative 3' UTR	Transcript name
si:ch211-241b2.1	ENSDARG00000044845.1	Novel protein annotated by Havana
IL1RAP	ENSDARG00000091088.1	uncharacterized protein
TRAF2 (1 of 2)	ENSDARG00000078378.1	TNF receptor-associated factor 2
CABZ01038499.1	ENSDARG00000088258.1	chromosome 16 open reading frame 52
ABTB1	ENSDARG00000063354.1	ankyrin repeat and BTB (POZ) domain containing 1
anks1b	ENSDARG00000003512.1	ankyrin repeat and sterile alpha motif domain containing 1B
ampd2	ENSDARG00000029952.1	adenosine monophosphate deaminase 2 (isoform L)
CABZ01045212.1	ENSDARG00000087525.1	Uncharacterized protein
fermt2	ENSDARG00000020242.1	fermitin family homolog 2 (Drosophila)
cdk12	ENSDARG00000063726.1	cyclin-dependent kinase 12
elp3	ENSDARG00000042005.1	elongation protein 3 homolog (S. cerevisiae)
arntl1b	ENSDARG00000035732.1	aryl hydrocarbon receptor nuclear translocator-like 1b
adcy7	ENSDARG00000060070.1	adenylate cyclase 7
SNX19 (1 of 2)	ENSDARG00000079931.1	Uncharacterized protein
SGSM2	ENSDARG00000063307.1	small G protein signaling modulator 2
ZNF507	ENSDARG00000052164.1	Uncharacterized protein
pex19	ENSDARG00000004891.1	peroxisomal biogenesis factor 19
tgfb1a	ENSDARG00000041502.1	transforming growth factor, beta 1a
fam69b	ENSDARG00000059881.1	family with sequence similarity 69, member B
ZEB1 (2 of 2)	ENSDARG00000016788.1	zinc finger E-box binding homeobox 1a
ERBB4 (3 of 5)	ENSDARG00000090408.1	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4

uhmk1	ENSDARG00000059575.1	U2AF homology motif (UHM) kinase 1
scpp1	ENSDARG00000090416.1	secretory calcium-binding phosphoprotein 1
CU856539.3	ENSDARG00000058949.1	spindlin family, member 2B
sall1a	ENSDARG00000074319.1	sal-like 1a (Drosophila)
nbeal2	ENSDARG00000057508.1	neurobeachin-like 2
mtx3	ENSDARG00000030390.1	metaxin 3
mtx3	ENSDARG00000090195.1	metaxin 3
C1H4orf27	ENSDARG00000057114.1	chromosome 4 open reading frame 27
mxd	ENSDARG00000023369.1	myxovirus (influenza virus) resistance D
gba2	ENSDARG00000061472.1	glucosidase, beta (bile acid) 2
psmb5	ENSDARG00000075445.1	proteasome (prosome, macropain) subunit, beta type, 5
bckdha	ENSDARG00000040555.1	branched chain keto acid dehydrogenase E1, alpha polypeptide
kbtbd5	ENSDARG00000039052.1	kelch-like 40a (Drosophila)
C5H9orf86 (2 of 2)	ENSDARG00000079046.1	RAB, member RAS oncogene family-like 6
FP236334.1	ENSDARG00000095664.1	Uncharacterized protein
lamp2	ENSDARG00000014914.1	lysosomal membrane glycoprotein 2
CR407701.1	ENSDARG00000089094.1	Uncharacterized protein
tpd52	ENSDARG00000061713.1	tumor protein D52
tmem2	ENSDARG00000061600.1	transmembrane protein 2
zbtb22b	ENSDARG00000003251.1	zinc finger and BTB domain containing 22b
arl3l1	ENSDARG00000028846.1	ADP-ribosylation factor-like 3, like 1

Table S5. Putative targets of miR-731 in the zebrafish genome predicted using the TargetScanFish Release 6.2 algorithm and ranked by their probability of conserved targeting (P_{CT}).

Target Gene	Representative 3' UTR	Transcript name
NBL1	ENSDARG00000031898.1	neuroblastoma 1, DAN family BMP antagonist
pfdn5	ENSDARG00000035043.1	prefoldin 5
ANK1 (1 of 2)|ank1	ENSDARG059093|092143.1	not present in the current release of the Ensembl database
rab23	ENSDARG00000004151.1	RAB23, member RAS oncogene family
UBAC2 (1 of 2)	ENSDARG00000060581.1	UBA domain containing 2
CABZ01045212.1	ENSDARG00000087525.1	Uncharacterized protein
pob	ENSDARG00000020607.1	ER membrane protein complex subunit 3
XPR1 (1 of 2)	ENSDARG00000062449.1	xenotropic and polytropic retrovirus receptor 1
si:dkeyp-120h9.1	ENSDARG00000020278.1	si:dkeyp-120h9.1
BX248318.1	ENSDARG00000022570.1	Uncharacterized protein
shpk	ENSDARG00000002355.1	sedoheptulokinase
cdc42l2	ENSDARG00000057869.1	cell division cycle 42 like 2
dcp1a	ENSDARG00000003323.1	DCP1 decapping enzyme homolog A (S. cerevisiae)
gpm6aa	ENSDARG00000055455.1	glycoprotein M6Aa
UBAC2 (2 of 2)	ENSDARG00000093065.1	UBA domain containing 2
CABZ01079302.1	ENSDARG00000091604.1	Uncharacterized protein
VAMP5	ENSDARG00000068262.1	vesicle-associated membrane protein 5

ldb2a	ENSDARG00000019579.1	LIM-domain binding factor 2a
whsc1l1	ENSDARG00000062765.1	Wolf-Hirschhorn syndrome candidate 1-like 1
apeh	ENSDARG00000008703.1	acylpeptide hydrolase
si:ch211-22n13.1	ENSDARG00000070528.1	calcium channel, voltage-dependent, T type, alpha 1H subunit b
lipea	ENSDARG00000063037.1	lipase, hormone-sensitive a
si:ch211-124k10.1	ENSDARG00000068731.1	relaxin/insulin-like family peptide receptor 2, like
IPO11	ENSDARG00000054224.1	importin 11
cttnbp2nl	ENSDARG00000056091.1	CTTNBP2 N-terminal like
onecutl	ENSDARG00000040253.1	one cut domain, family member, like
smtnl1	ENSDARG00000041257.1	smoothelin-like 1
bcas3	ENSDARG00000090764.1	breast carcinoma amplified sequence 3
FUT11	ENSDARG00000057727.1	fucosyltransferase 11 (alpha (1,3) fucosyltransferase)
rab34b	ENSDARG00000010977.1	RAB34, member RAS oncogene family b
mpg	ENSDARG00000069729.1	N-methylpurine-DNA glycosylase
slc4a4a	ENSDARG00000013730.1	solute carrier family 4, member 4a
ccdc6a	ENSDARG00000043334.1	coiled-coil domain containing 6a
fam155a	ENSDARG00000075858.1	family with sequence similarity 155, member A
erbb4a	ENSDARG00000063207.1	v-erb-a erythroblastic leukemia viral oncogene homolog 4a (avian)
si:ch211-217k17.10	ENSDARG00000079345.1	si:ch211-217k17.10
tom1	ENSDARG00000043638.1	target of myb1 (chicken)
CR848759.2	ENSDARG00000079119.1	si:ch211-229d2.5
PACS1 (1 of 3)	ENSDARG00000019800.1	phosphofurin acidic cluster sorting protein 1
birc7	ENSDARG00000058082.1	baculoviral IAP repeat-containing 7

Text S1. Supplementary Materials and Methods

Viral challenge experiment

Two groups of 2x20 5g disease-free rainbow trout were kept in aquaria at 12˚C (experimental temperature). Each aquarium contained 8 litres of water, which was continuously renewed by a water flow-through system. The water flow-through was stopped and VHSV isolate DK-3592B (55) was added to one of the experimental aquaria (the other serving as negative control) to give a final concentration of approximately 10^5 $TCID_{50}$/ml**.** After one hour of viral challenge the water flow-through was restarted. Throughout the next week, dead fish were daily removed from the challenge-aquarium, counted, examined for external signs of disease such as bleedings in eyes and skin, swollen belly, swollen eyeballs, and dark skin. Liver samples for RNA purification were taken from fish with external signs of disease day 7 (5 fish) and day 8 (4 fish). Both days the same number of fish was sampled from the negative control aquarium. Prior to sampling, fish were anaesthetized in benzocaine (ethyl p-aminobenzoate; Sigma; cat.no.E-1501) diluted to 0.01%. Verification of VHSV infection in the diseased fish was carried out by the use of a previously published ELISA on organ samples (55).

Microarray analysis of miRNA regulation

Before the microarray analysis RNA samples were quality tested by verifying the presence of 28s rRNA, 18s rRNA and tRNA fractions after running for 1-2 hours (70 volts) in a 1% agarose gel containing 2% formaldehyde and stained using SyberGold

(Invitrogen, USA). Furthermore, quantities and purity of samples were re-measured by nanodrop as described above. The microarray for detecting miRNA levels was run by a microarray profiling service (Ocean Ridge Biosciences, USA) using the Ncode Version 2.0 probe set (Invitrogen) containing 1492 unique probes including 218 zebrafish probes (35-44-mer oligoes spotted in triplicates) for detection of all miRNAs in the Sanger Institute database miRBase version 9.0 (Nov´06). Two hundred ng RNA from each sample labeled by ligation of DNA dendrimer containing 15-fluorophores to 3´-OHs of microRNAs (Genisphere, USA). One-way ANOVA was used to assure that the overall intensity difference between infected and non-infected fish was significant. Raw data were $\log_2$ transformed and normalized to the mean signal of all detected probes (120 probes which were detectable both among the infected and control samples. Triplicate spots were averaged throwing out flagged spots. Data from infected versus control samples for the 120 probe spots were compared using the NIA Array Analysis Software (56).

cDNA synthesis

1 μg of total RNA was used for cDNA synthesis by the QuantiMir Synthesis Kit (System Biosciences, USA, cat. no. RA420A-1) following manufacturer's instructions. Briefly, RNAs were poly-A tailed using poly-A polymerase. The poly-A tail allows for the binding of an oligo-dT adaptor, which contains a universal reverse primer site. Following reverse transcription from this adaptor, a cDNA pool was generated, which contains strands complementary to various RNAs in the original samples and the universal reverse primer site at the distal 3´end.

Genomic DNA extraction and generation of PCR products for sequencing

Genomic DNA was extracted from the liver tissue of disease-free rainbow trout using DNeasy Blood & Tissue Kit (Qiagen, Germany) following manufacturer's instructions. The DNA was eluted in AE Buffer (10 mM Tris·Cl, 0.5 mM EDTA, pH 9.0.) and stored at -20 °C. Three conserved sequences were found by aligning fish genomic sequences, and used to design primers to generate two PCR products. The primer pair used for PCR product 1: forward primer, 5'-GTAACGGAACCCATAATGCAGCT-´3 and reverse primer 5'- CTTGGCTGACACGAAITTCCCGGT-'3. PCR product 2 was amplified with forward primer, 5'- AGAAGTGAAAGTGAAA-3' and reverse primer 5'-CAGCTGCATTATGGGTTCCGTTAC- 3'.

MANUSCRIPT 2.

Involvement of two microRNAs in the early immune response to DNA vaccination against a fish rhabdovirus

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To be submitted.

Involvement of two microRNAs in the early immune response to DNA vaccination against a fish rhabdovirus

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ABSTRACT

Mechanisms that account for the high protective efficacy of a DNA vaccine expressing the glycoprotein (*G*) of *Viral hemorrhagic septicemia virus* (VHSV) are thought to involve early innate immune responses mediated by interferons (IFNs). In order to reveal the complex web of interactions involved in the underlying immune response, key factors must be identified. Microribonucleic acids (miRNAs) are a diverse class of small (18-22 nucleotides) endogenous RNAs that potently mediate post-transcriptional silencing of a wide range of genes and are emerging as critical regulators of cellular processes, including immune responses. We have recently reported that two miRNAs, miR-462 and miR-731, which have been only described in teleost fish, were induced in rainbow trout infected with VHSV. In this study, we analyzed the expression of these miRNAs in fish following administration with the DNA vaccine.

Quantitative RT-PCR analysis revealed the increased levels of miR-462, and miR-731 in the skeletal muscle tissue at the site of vaccine administration and in the liver of vaccinated fish relative to empty plasmid backbone-injected controls. The increased expression of these miRNAs in the skeletal muscle correlated with the increased levels of the type I interferon (IFN)-inducible *Mx* gene, the vaccine gene (*G*), type I IFN and IFN-γ genes at the vaccination site. Intramuscular injection of fish with either type I IFN or IFN-γ construct resulted in the upregulation of miR-462 and miR-731 in the site of injection relative to controls, further suggesting that the induction of these miRNAs is elicited by IFNs. Silencing miR-462 and miR-731 with specific anti-miRNA oligonucleotides in poly I:C-treated rainbow trout fingerlings followed by VHSV challenge demonstrated faster disease development and higher mortalities in anti-miR-injected fish relative to controls. Counteracting poly I:C-induced miR-462/731

expression and protective effect by specific anti-miRNAs indicates that miR-462/731 may be involved in IFN-mediated protection conferred by poly I:C and the rhabdovirus G-based DNA vaccine and in the antiviral response in teleosts in general.

Keywords: microRNA, *Viral hemorrhagic septicemia virus* (VHSV), DNA vaccination, interferons, rainbow trout

INTRODUCTION

Viral hemorrhagic septicemia caused by the fish rhabdovirus *viral hemorrhagic septicemia virus* (VHSV) results in mortality as high as 90% in farmed fish (Olesen, 1998), causing huge economic loss. The presence of a marine wildlife reservoir of VHSV poses a potential threat to sea-farmed fish and necessitates the development of effective disease management strategies. Fish rhabdovirus DNA vaccines encoding the glycoprotein G of VHSV and *infectious hematopoietic necrosis virus* (IHNV) have been previously shown to confer efficient protection against lethal virus challenge under laboratory conditions (Lorenzen and LaPatra, 2005; Lorenzen et al., 1998). The immune response elicited by these vaccines involves early, non-specific, and short-term immunity, followed by specific, long-lasting protection (Kurath, 2008; Lorenzen and LaPatra, 2005). Neutralizing antibodies as well as cytotoxic cells are believed to be involved in the specific protection (Utke et al., 2008; Lorenzen and LaPatra, 2005; Boudinot et al., 1999). The early protection correlates with upregulation of the interferon (IFN)-induced antiviral myxovirus resistance Mx protein but the exact protective mechanism remains to be elucidated (Kurath, 2008; Kurath et al., 2006; McLauchlan et al., 2003; Lorenzen et al., 2002; Lorenzen et al., 1999). Type I IFN responses implicate an activation of an array of innate antiviral defense mechanisms and recent reports suggest that these also include small regulatory RNAs known as microRNAs (miRNAs) (Sisk et al., 2013; Russo and Potenza, 2011; Pedersen et al., 2007).

MicroRNAs are small (19-25 nucleotides), endogenously expressed single-stranded RNAs that post-transcriptionally regulate gene expression (Bartel, 2004). By repressing translation and/or degrading target mRNAs, miRNA-mediated RNAi results in altered

mRNA and protein expression profiles (Bartel, 2004). Each miRNA is believed to regulate multiple genes (Creighton et al., 2012; Bonci et al., 2008), and as such could steer different mechanisms in various cell and tissue types, modulating diverse pathways for fine-tuning physiological conditions and defining distinct cell types (Smigielska-Czepiel et al., 2014; Allantaz et al., 2012; Chen et al., 2004). By targeting numerous mRNAs (Friedman et al., 2009; Lim et al., 2005), miRNAs can control a broad spectrum of cellular processes, ranging from cell differentiation and development to immune responses, thus providing an additional layer to the molecular variety of gene regulatory mechanisms.

Whereas the exact global functional outcomes of the expression of a particular miRNA often remains to be determined, they have been shown to be involved in development and function of immune cells (Wu et al., 2007) and host-pathogen interactions (Staedel and Darfeuille, 2013; Swaminathan et al., 2013; Zhang and Li, 2013; Eulalio et al; 2012; Skalsky and Cullen, 2010; Ghosh et al., 2009).

We have recently reported that two miRNAs known only in teleost fishes, miR-462 and miR-731, were the most highly induced miRNAs in the liver of rainbow trout following VHSV infection (Schyth et al., manuscript submitted). Here, we report the upregulation of these two miRNAs in fish immunized with a VHSV glycoprotein-expressing DNA vaccine and determined the kinetics of expression following vaccination. We show that the expression of these miRNAs is elicited by IFN induction. Finally, inhibition of these two miRNAs in fish treated with the TLR3 agonist and IFN inducer, poly I:C, reduced the ability of poly I:C to protect fish against lethal VHSV infection.

MATERIALS AND METHODS

Fish and virus. Outbred rainbow trout eggs were obtained from a commercial fish farm certified as disease-free. Fish were hatched from disinfected eggs and kept in pathogen-free aquarium facilities until injection experiments and infection trials. Fish were acclimatized to laboratory conditions several weeks prior to the experiments. Throughout the experiments, fish were maintained in 8 L aquaria at 10 ± 2 °C supplied with flow through partly deionized water. The VHSV isolates DK-3592B (Lorenzen et al., 1993) or a Norwegian isolate were used in the infection trials.

DNA vaccine. The DNA vaccine (pcDNA3-vhsG) consists of the VHSV glycoprotein gene inserted under the control of the cytomegalovirus (CMV) promoter at a multiple cloning site in a commercially available plasmid, pcDNA3 (Invitrogen) as described previously (Lorenzen et al., 1998). Control fish were injected with either pcDNA3 (empty vector) diluted in 0.9% NaCl (physiological saline) or physiological saline only.

IFN-expressing plasmids. The IFN constructs consist of the IFN gene under the control of the cytomegalovirus (CMV) promoter at a multiple cloning site in a commercially available plasmid, pcDNA3.1/3.3 (Invitrogen) and were kindly supplied by Dr. J. Zou (Zou et al., 2007; Zou et al., 2005). The constructs are herein referred to as pcDNA3.3-IFN 1-13, and pcDNA3.1-IFN-G. Control fish were injected with pcDNA3.1 (empty vector) dissolved in 0.9% NaCl (physiological saline) or with physiological saline only.

Poly I:C and anti-miRNAs. Polyinosinic: polycytidylate sodium salt (Poly I:C; CAS # 42424-50-0) was purchased from Sigma-Aldrich GmBH (Steinheim, Germany). The anti-miR-462 and anti-miR-731 and their corresponding mismatch controls were

unconjugated 2'-O-methylated Locked Nucleic Acid (LNA)-based oligonucleotide probes synthesized by RiboTask (Langeskov, Denmark). The anti-miRs were designed as complete anti-sense sequences of the mature zebrafish miRNA sequences, whereas the corresponding mismatch anti-miRNA controls differed from the anti-miRNAs by two nucleotides (underlined bases) within the anti-seed region (Table 1). Positions in which the control anti-miRNAs differed from the anti-miRNAs are underlined and are located in positions complementary to that of the seed sequence in the mature miRNAs. 2'-O-methylated positions are indicated/preceded by the letter m, while the LNA coupled positions are indicated by the letter I.

Table 1. Anti-miRNAs and mismatched controls used in the experiments.

Name	Sequence (5'→3')
dre-anti-miR-462 probe	mA mU IT mA mU IG mG mG I<u>T</u> mU mC I<u>C</u> mG mU IT mA
dre-anti-miR-731 probe	mG mA IG mA mA IA mA mC I<u>G</u> mU mG I<u>T</u> mC mA IT mU
dre-anti-miR-462 mismatch probe	mA mU IT mA mU IG mG mG I<u>A</u> <u>m</u>U mC I<u>A</u> <u>m</u>G mU IT mA
dre-anti-miR-731 mismatch probe	5'mG mA IG mA mA IA mA mC I<u>C</u> <u>m</u>U mG I<u>A</u> <u>m</u>C mA IT mU

Vaccination and injection with IFN constructs. Fish anaesthetized with 0.01% benzocaine were injected with 10 µg of pcDNA3-vhsG or 10 µg of pcDNA3 empty vector plasmid backbone in 20 µL phosphate buffered saline (PBS) or PBS only in the right, epaxial muscle in front of the dorsal fin as described by Lorenzen et al. (2009). For injection with IFN-expressing plasmids, benzocaine-anaesthetized fish were

injected with 10 µg of each of the above-mentioned constructs in 20 µL PBS, or 5 µg poly I:C (Sigma) in 20 µL PBS. Negative controls were injected with 10 µg of the empty vector in 20 µL PBS or 20 µL PBS alone.

At 1, 4, 7, and 21 days post-vaccination (dpv), muscle tissue from the injection site and liver from 6 fish were sampled for each group. Muscle tissue was immediately processed for RNA isolation while liver samples were stored at -80C in RNALater until needed.

RNA Isolation and cDNA Synthesis. Total RNA was isolated and purified from tissue samples using the miRNeasy Kit spin column purification (cat. # 217004, Quiagen SA Biosciences, Hilden, Germany), following manufacturer's protocol for animal tissues with DNase treatment (Quiagen RNase-free DNase Set, cat. # 79254).

For miRNA expression studies, total RNA (1 µg) was used to reverse transcribe and convert small non-coding RNAs into quantifiable cDNAs with the QuantiMiR RT Kit Small RNA Quantitation System (cat. # RA420A-1, System Biosciences, SBI, Mountain View, CA, USA) following manufacturer's instructions. For mRNA (*Mx, IFN-1, IFN-G*) expression analyses, 1 µg RNA was used to make cDNA using iScript cDNA synthesis kit from BioRad (cat. # 170-8891, Hercules, CA, USA) following manufacturer's instructions.

Quantitative RT-PCR for miRNAs and mRNAs. MicroRNA-specific forward primers together with a 3' Universal reverse primer (supplied in the QuantiMiR RT Kit; designed to hybridize with the anchor tail in the oligo-dT adaptor used in cDNA synthesis) were used in quantitative real time PCR to detect miRNA expression. Forward miRNA-specific primers were exact mature sequences of the zebrafish miRNAs of interest converted to DNA. The transcript levels of the IFN-induced anti-

viral protein Mx, type I IFN, IFN-gamma were also quantified. The miRNA expression levels were normalized to that of the internal control dre-snoU23 transcript, whereas the level of *Mx* mRNA was normalized to the level of acidic ribosomal protein (*ARP*) mRNA in the same sample. Primer sequences for both miRNA and mRNA expression analysis are given in Table 2.

Reactions were run on an Mx3005P machine (Stratagene) in final reaction volumes of 15 µL containing 5 μl diluted cDNA template (1:100), 333 *n*M miRNA-specific forward primer, 167 *n*M universal reverse primer, ROX reference dye, water, and 7.5 μL of SYBR Green PCR master mix (Brilliant SYBR Green qPCR Master Mix, Cat. No. 600548, Stratagene, La Jolla, CA, USA). The program was set to an initial denaturation step for 5 minutes at 95^0C and 40 cycles of denaturation for 20 seconds at 95^0C and annealing/extension for 20 seconds at 65^0C, with fluorescent data collection. To get melting curve data, PCR amplicons were denatured for 1 minute at 95^0C followed by a ramp down to 55^0C for 30 seconds and then a gradual increase to 95^0C at 0.2^0C/second, with continuous fluorescence recording for making a dissociation curve. Transcript levels were measured as quantification cycle (Cq) values and normalized to that of the corresponding internal reference genes. Values presented are normalized Cq values from 6 fish. Negative control (without template) reactions were also carried out for each primer pair. The relative quantity values of miRNA or mRNA expression signals were calculated from the mean values of duplicate measurements using the $\Delta\Delta C_t$ method, where Relative Expression = $2^{-\Delta\Delta C}{}_t$ and $\Delta\Delta C^t = (C_{t\ \text{experimental}} - C_{t\ \text{reference gene}}) - (C_{t\ \text{control}} - C_{t\ \text{reference gene}})$. The sizes of all PCR amplicons were verified by inspection of the dissociation curve and/or by agarose gel electrophoresis.

Efficiency of amplification was determined for each primer pair using 2-fold dilutions (100, 200, 400, 800, and 1600). A sample from the serial dilution was resolved

on an agarose gel and visualized under UV light after staining with ethidium bromide, to verify the amplification of a single band of the correct size.

Table 2. Primers used in real time PCR analysis

Gene name	Accession number	Primer sequence (5'→ 3')
dre-miR-462	MIMAT0001855	Forward TAACGGAACCCATAATGCAGCT
dre-miR-731	MIMAT0003761	Forward AATGACACGTTTTCTCCCGGATCG
dre-snoU23	AJ009730	Forward GCCCATGTCTGCTGTGAAACAAT
omy-Mx3	U47946	Forward GCAGAGGGAGATGCTTCAGA Reverse CGATGTCAAAGTCCTCCTTCA
omy type I IFN	AY788890.1	Forward GAAAAGGACTGGGGCATTCT Reverse GAATACCTTTCCCTGCTGGAC
IFN Gamma	AY795563	Forward AAGGGCTGTGATGTGTTTCTG Reverse TGTACTGAGCGGCATTACTCC
omy-ARP		Forward TCTACAACGAGCTGCGAGTG Reverse GGGGTGTTGAAGGTCTCAAA

Anti-miRNA injection and virus challenge experiments: Experiment 1. All groups were in duplicate aquaria (17-21 fish/aquarium). Rainbow trout fingerlings (approx. 1 g) were anaesthetized in water with benzocaine. Based on the treatment groups, fish were injected intra-peritoneally (IP) with dre-anti-miR-462/dre-anti-miR-731 probe mix (0.5 µg/g of each anti-miR in 20 µL TE buffer per fish), dre-anti-miR-462/dre-anti-miR-731 mismatch probe mix (0.5 µg/g of each anti-miR in 20 µL TE buffer per fish), 5 µg poly I:C in 20 µL TE buffer (control), or 20 µL of TE buffer only (control). Water flow was discontinued 24 hours after anti-miRNA administration and the day of virus

challenge. Depending on the virus challenge group, VHSV isolate DK-3592B (high virulence strain; Lorenzen et al., 1993) or Norwegian VHSV isolate NO-2007-50-385 NR1 (semi-virulent strain) was added to each experimental aquarium to a final titer of approximately 10^4 tissue culture infectious $dose_{50}$ ($TCID_{50}$)/mL per aquarium. Virus challenge lasted for 2 hours, after which water flow was restarted. Unchallenged controls consisted of a mix of fish from each of the treatment groups. Disease development in each aquarium was monitored daily for 14 days post-challenge (dpc) for the group challenged with the high virulence strain or 32 dpc for the group challenged with the semi-virulent strain. Moribund/dead fish were terminated, removed, registered, examined for external manifestations of disease (skin darkening, bleeding, ascitis or swollen belly, exopthalmia or swollen eyeballs) and frozen (for virological examination).

Anti-miRNA injection and virus challenge experiments: Experiment 2. All groups were in duplicate aquaria (18-27 fish/aquarium). In contrast to experiment 1, fish were treated with poly I:C prior to anti-miRNA administration. Rainbow trout fingerlings (approx. 0.5 g) anaesthetized in benzocaine were injected IP with 5 µg poly I:C in 20 µL physiological saline or physiological saline only (control). 24 hours post-poly I:C treatment or saline injection, fish grouped accordingly were injected IP with a first dose of dre-anti-miR-462/dre-anti-miR-731 probe mix (1 µg/g of each anti-miR in 20 µL TE buffer per fish), dre-anti-miR-462/dre-anti-miR-731 mismatch probe mix (1 µg/g of each anti-miR in 20 µL TE buffer per fish), or TE buffer only (control). A second dose of anti-miR mix, mismatch control mix, or TE buffer (same dose as the first) was administered IP 24 h post-first dosing. Water flow was discontinued 24 hours after the second treatment dose and the day of virus challenge. Only VHSV isolate DK-3592B (Lorenzen et al., 1993) was used for virus challenge in this experiment, which was

added to each experimental aquarium to a final titer as in experiment 1. Virus challenge was done for 2 hours and water flow was subsequently restarted. Unchallenged controls consisted of a mix of fish from each of the treatment groups. Disease development in each aquarium was monitored daily for 14 dpc. Moribund/dead fish were handled as described earlier.

Virological examination. Fish organs (brain, head kidney, and spleen pooled according to treatment group and aquarium) were homogenized with a tissue lyser. The homogenates were treated with gentamycin (25 μL mL^{-1}) and kept overnight at 4 °C, after which were centrifuged at approximately 16 000 x***g*** for 15 min. Three-fold dilutions (1×3^{-1} to 1×3^{-3}) of the resulting supernatant were prepared and inoculated onto BF2 cell monolayers grown to 60–80% confluence in 24-well plates and incubated at 15 °C. After 7 days, supernatants were sampled and analyzed by a VHSV-specific enzyme-linked immunosorbent assay (ELISA) (Mortensen et al., 1999). Samples giving reactions of 3 times the background levels (cell culture supernatant from non-inoculated cells) were considered positive.

RESULTS

miR-462 and miR-731 were upregulated in pcDNA3-vhsG-vaccinated rainbow trout

The expression levels of these two microRNAs were upregulated (relative to empty vector-injected controls) in the skeletal muscle (at site of vaccination) and in the liver of rainbow trout following vaccination with pcDNA3-vhsG (Figure 1A and 1B). Levels of miR-462 and miR-731 expression increased 4 days post-vaccination (dpv), which continued to 3 weeks. The expression of the two miRNAs in both tissues showed a similar kinetics (Figure 1A and 1B).

Transcript levels of the type 1 IFN-inducible myxovirus resistance (*Mx*) gene also increased in both the site of vaccination and in the liver at 4 dpv (Figure 2A). *Mx* expression levels decreased with time but remained upregulated up to the final sampling time point (21 dpv) (Figure 2A). Likewise, the levels of type I IFN and IFN-γ transcripts were also found to increase at the vaccination site (Figure 2B).

miR-462 and miR-731 were induced in fish injected with type I and type II IFN-expressing plasmid constructs

Increased expression of miR-462 and miR-731 in the skeletal muscle tissue (at injection site) was also observed in fish injected with plasmid constructs encoding a type I IFN (pcDNA3-IFN 1-13) or IFN-γ (pcDNA3-IFN-G) relative to controls (Figure 3A). As positive control, the general IFN stimulator and TLR3 agonist poly I:C, upregulated miR-462/731 expression (Figure 3A). The levels of *Mx* transcripts were likewise induced in the injection site following administration with the IFN constructs and poly I:C (Figure 3B).

Effects of miR-462 and miR-731 inhibition with specific anti-miRNAs in fish

Inhibitory Locked Nucleic Acid (LNA) antagomiR (anti-miR)-462 and anti-miR-731, anti-sense oligonucleotides that hybridize with miR-462 and miR-731, respectively, were designed in order to determine the potential role of these miRNAs in the interaction between the fish host and VHSV. The LNA oligonucleotides did not stimulate the IFN response (data not shown).

***Anti-miRNA injection followed by lethal virus challenge did not show significant effect on the development of disease and mortality*.** In a first experiment, fish were injected IP with anti-miRNAs followed by challenge with highly virulent or semi-virulent VHSV strains. Injection with anti-miRs, mismatch controls, or with saline/TE showed similar cumulative mortality profiles reaching 90% 14 days post-challenge (dpc) with the highly virulent DK3592 VHSV strain (Figure 4A). On the other hand, poly I:C injection resulted in lower mortality (~40% mortality 14 dpc), indicating the protective effect of poly I:C (Figure 4A).

Challenge with the semi-virulent Norwegian VHSV strain showed slow disease progression and resulted in generally low mortalities. The groups injected with poly I:C, anti-miR mismatch controls, or with saline/TE showed similar mortality curves, whereas the group injected with anti-miRNAs showed higher mortality, although still low at 16% 32 dpc (Figure 4B).

***Anti-miRNA treatment of poly I:C-treated fish accelerated disease progression and increased mortality*.** In the second experiment, fish pre-treated with poly I:C were injected with anti-miRNAs or mismatch controls and subsequently challenged with the highly virulent VSHV strain. Injection with poly I:C gave protection by slowing down

the development of disease and decreasing mortality (60% mortality, compared with almost 100% cumulative mortality 21 dpc in fish that did not receive poly I:C) (Figure 5). Administration with a mixture of anti-miR-462/731 to poly I:C-treated fish hastened disease progression and increased mortality compared to the poly I:C-treated group that was not injected with anti-miRNAs. In fish that did not receive poly I:C treatment, injection with anti-miRNAs only or with mismatch controls only showed similar disease development and mortality profiles with the group injected only with saline/TE.

The mortality among fish injected with mismatch anti-miRNA controls following poly I:C treatment varied significantly between the replicate aquaria, with only one moribund fish in one replicate.

Virological examination

Dead/moribund from challenge experiments as well as surviving fish from unchallenged controls were examined for the presence of VHSV by cell culture inoculation followed by ELISA on cell culture supernatants harvested 7 days post-inoculation.

In experiment 1, homogenates from pools of organs from dead/moribund fish from the challenged groups all tested positive for VHSV in ELISA (Tables 3). In experiment 2, tissue homogenates from dead/moribund fish all tested positive for VHSV in ELISA (Table 4).

Table 3. Virological analysis of organs from fish injected with anti-miRNAs and challenged with VHSV (experiment 1).

	No challenge	Challenge							
		DK-3592B Isolate				Norwegian isolate			
		TE-Saline	mismatch controls	anti-miRs	Poly I:C	TE-Saline	mismatch controls	anti-miRs	Poly I:C
Moribund/ dead fish (%)	0;	89.5;	94.7;	100;	47.4;	0;	5.0;	21.1;	15.8;
Repl. 1; Repl. 2	0	84.2	84.2	89.5	52.6	14.3	17.6	11.1	5.3
ELISA for VHSV	Neg;	Pos;	Pos;	Pos;	Pos;	Neg;	Pos;	Pos;	Pos;
Repl. 1; Repl. 2	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Table 4. Virological analysis of organs from poly I:C pre-treated fish injected with anti-miRNAs and challenged with VHSV (experiment 2).

	Unchallenged	Treatments					
		+ poly I:C			- poly I:C		
		TE-Saline	mismatch control	anti-miRNAs	TE-Saline	mismatch control	anti-miRNAs
Moribund/dead fish (%)	0;	54.5;	5;	100;	100;	100;	100;
Repl. 1; Repl. 2	0	60.7	38.1	86.4	94.4	96	100
ELISA for VHSV	Neg;	Pos;	Pos;	Pos;	Pos;	Pos;	Pos;
Repl. 1; Repl. 2	Neg	Pos	Pos	Pos	Pos	Pos	Pos

Neg = negative for VHSV in ELISA

Pos = positive for VHSV in ELISA

DISCUSSION

We have recently reported that the clustered miRNAs miR-462 and miR-731 known so far only in teleost fishes (Xu et al., 2013; Li et al., 2010; Kloosterman et al., 2006; Chen et al., 2005) were the most highly upregulated miRNAs in rainbow trout liver infected with VHSV (Schyth et al., manuscript submitted). In this study we report that miR-462 and miR-731 are also highly induced in fish immunized with the protective VHSV glycoprotein-based DNA vaccine, pcDNA3-vhsG, and that the two miRNAs contribute to the protective mechanisms induced by the IFN stimulator poly I:C.

The parallel miR-462 and miR-731 induction and expression kinetics in both vaccination site and the liver reflects their genome organization. The two miRNAs are members of a cluster (miR-462/731 cluster) located in an intergenic region flanked by inosine monophosphate dehydrogenase 2 (*IMPDH2*) and DALR anticodon binding domain 3 (*DALRD3*) genes in teleost fish genomes, including rainbow trout (Schyth et al., manuscript submitted). The miRNAs in the cluster are most likely expressed as part of a polycistronic transcript, as are other clustered miRNAs.

The induction of miR-462 and miR-731 following the administration of the DNA vaccine (this study) and in VHSV-infected fish (Schyth et al., manuscript submitted) may be attributed to the stimulation of the IFN response elicited by immunization with the VHSV-G-based DNA vaccine (Byon et al., 2006; Byon et al., 2005) as well as by VHSV infection (Verrier et al., 2011; Bernard et al., 1985), respectively. Fish rhabdovirus glycoproteins, including VHSV-G, has been known to be a potent elicitor of the IFN response (Chen et al., 2012; Verjan et al., 2008; Acosta et al., 2006; Lorenzen et al., 2002; LaPatra et al., 2001). This was here confirmed by the increased levels of type I IFN and IFN-γ transcripts at the vaccination site (Figure 2B).

Induction of the two miRNAs in both the site of vaccination and in the liver also correlated with increased transcript levels of the type 1 IFN-inducible *Mx* gene, which remained upregulated to the final sampling time point (21 dpv) (Figure 2A). The Mx protein is known as a non-specific antiviral protein in mammals (Jin et al., 1999) and in other vertebrates, including teleost fishes (Verhelst et al., 2013; Trobridge and Leong, 1995) and has earlier been shown to be upregulated in fish injected with the plasmids mediating the expression of the rhabdovirus G protein (Kim et al., 2000; Boudinot et al., 1998). In teleost fishes, the expression of *Mx* is stimulated both by IFN-α (a type I IFN) (Berg et al., 2009; Robertsen et al., 2003) and to a less extent by IFN-γ (Sun et al., 2011). Taken together, results indicate that injection of pcDNA3-vhs-G induced a systemic IFN response, which in turn stimulated the expression of miR-462 and miR-731.

This was further supported by the observation that injection of fish with type I- or type II IFN-encoding plasmid constructs or with the IFN-stimulator and TLR3 agonist poly I:C (Richmond and Hamilton, 1969), resulted in upregulated levels of miR-462, miR-731, as well as *Mx* transcripts at the site of injection. Upregulation of miR-462/731 levels following poly I:C treatment in cultured rainbow trout liver cells has been previously reported (Schyth et al. manuscript submitted). A highly conserved IFN-stimulated response element (ISRE) was found in the promoter upstream of the miR-462/731 cluster locus in teleost fish (Schyth et al., manuscript submitted), accordingly supporting the stimulatory role of IFN.

Which cell types that express miR-462 and miR-731 are currently not known. At the vaccination site the two miRNAs may have been induced in myocytes due to IFN induction following vaccine injection. Alternatively, miR-462 and miR-731 could be induced in immune cells infiltrating the site of vaccination. Immunization with

pcDNA3-vhs-G vaccine has been shown to promote the recruitment of immune cells at the site of vaccine administration (Castro et al., 2014; Utke et al., 2008; Lorenzen et al., 2005; Lorenzen et al., 2002). The increased expression of IFN-γ observed at the site of vaccination accordingly suggested the presence of activated NK cells, NKT cells, and/or T lymphocytes.

Whereas the kinetics of miR-462/731 and *Mx* expression timely correlated in terms of onset, levels of miRNA transcripts remained particularly elevated despite the relative decrease of *Mx* expression 21 dpv (Figure 1A,B). This suggests that in addition to the putative roles of miR-462/731 in the early innate immune response, they may potentially also be involved in the development of specific adaptive immune responses such as the activation and differentiation of immune cells. Apart from the ISRE element, the miR-462/731 promoter region thus also contained a gamma IFN activation sequence (GAS) and a purine box 1 (PU.1 box: GAGGAAGT) (Schyth et al., manuscript submitted). The PU.1 box, a conserved immune-related regulatory element in vertebrate (including teleost) genomes, is the binding site of the leukocyte differentiation related transcription factor PU.1, which is involved in the regulation of hematopoiesis (Mak et al., 2011; Ribas et al., 2008; Anderson et al., 2001; Shintani et al., 2000; DeKoter et al., 1998). Isolation of infiltrating cells at the vaccination site and subsequent miRNA profiling will be needed to resolve this.

Injection of the anti-miRNA-462/731 before challenge of the fish with VHSV tended to promote the disease progression (Figure 5). However, the effect was minor and not conclusive, possibly due to an excessive challenge dose with the highly virulent VHSV isolate and an insufficient challenge dose with the low virulent isolate. Alternatively, the persistence of the anti-miRNAs might have been too short to clearly affect the miRNAs induced by the subsequent VHSV infection. To increase the chance of having

anti-miRNAs present in the fish at the time of miRNA induction, we therefore decided to test the effect of anti-miRNAs on poly I:C induced protection against VHSV.

Poly I:C has been previously shown to induce the IFN response in teleost fish and protects against virus infection *in vitro* (Verrier et al., 2013) and *in vivo* (Nishizawa et al., 2009; Jensen et al., 2002; Eaton, 1990) shortly after administration, as was also demonstrated in this study (Figures 4A and 5). Fish pre-treated with poly I:C were injected with anti-miRNAs or mismatch controls and subsequently challenged with the highly virulent VSHV strain.

Poly I:C treatment slowed down disease progression and decreased mortality 21 dpc relative to non-poly I:C-treated groups (Figure 5). Anti-miR-462/731 injection in poly I:C-treated fish led to faster disease development and increased mortality compared to the poly I:C-treated group that did not receive anti-miRNAs. This demonstrates that silencing miR-462/731 with anti-miRNAs counteracted the protective effects of poly I:C against VHSV. Anti-miRNA or mismatch control injection to non-poly I:C-treated groups showed similar disease development and mortality profiles as the saline/TE-injected controls. In the poly I:C-treated fish receiving mismatch controls, challenge failed in one of the replicate tanks. Mortality comparable to the poly I:C-treated fish tanks in the remaining replicate suggested a specific inhibitory effect of the anti-miRNAs.

One basic question related to the observed effect of the anti-miRNAs on virus susceptibility is whether the anti-miRNAs are systemically distributed and taken up by cells following IP delivery. Efficient delivery remains one of the major challenges in the development of nucleic acid-based medicines (Hsu and Uludağ, 2012; Jafari et al., 2012; Lee and Kataoka, 2012, Xu and Anchordoquy, 2011; Mastrobattista et al., 2007).

Reports on systemic effects of antisense oligonucleotides, i.e. anti-miRNAs, in mammals suggest that it is possible to specifically inhibit miRNAs *in vivo* by the administration of small chemically-modified, unconjugated or cholesterol-conjugated or naked LNA-based anti-miRNAs (Bernardo et al., 2014; Bernardo et al., 2012; Hildebrandt-Eriksen et al., 2012; Lanford et al., 2010; Elmen et al., 2008; Esau et al., 2006; Krützfeldt et al., 2005). Furthermore, a short LNA-based anti-miRNA against miR-122 is currently in phase 2 clinical trials for the treatment of HCV infection (Janssen et al., 2013). Novel data demonstrate that this LNA-modified DNA phosphorothioate antisense oligonucleotide drug called Miravirsen not only blocks the mature miR-122 but also interferes with precursor and primary miRNA stages, thereby interrupting miRNA biogenesis and the production of the functional mature miRNA (Gebert et al., 2014). Although the specific interference with miR-462/731 by the administered anti-miRNAs at the molecular level remains to be confirmed from the work presented here, current research thus supports that endogenously expressed miRNAs can be specifically inhibited by the applied approach.

Altogether, the fish trials suggest that the two miRNAs are involved in IFN-mediated antiviral activities. IFNs are important mediators of immune responses that are elicited by the recognition of virus pathogen-associated molecular patterns (PAMPs) by host cell receptors. Type I IFNs induce the expression of the cells' predominantly protein-based antiviral resources including the Mx GTPases, adenosine deaminase (ADA), protein kinase R (PKR), and the 2'-5' oligoadenylate synthetase (OAS)/RNAse L system, (Schoggins and Rice, 2011; Borden et al., 2007; Samuel 2001). Although IFNs have been shown to upregulate expression of cellular miRNAs in human cells (Zhang et al., 2013; Scagnolari et al., 2010), the role of these miRNAs, particularly in host-pathogen interactions, has not been well established.

Cellular miRNAs induced by IFN-β in human cells have been demonstrated to target viral transcripts, thereby contributing towards the antiviral properties of IFN-β (Pedersen et al., 2007). Similarly, an IFN-γ-induced miRNA, miR-29a (Schmitt et al., 2012) has been shown to target the 3'UTR of HIV-1 (Nathans, et al., 2009). The recent years have also seen an increasing number of cellular miRNAs reported to be involved in antiviral responses against diverse viruses in mammalian cells (Russo and Potenza, 2011). Some of these miRNAs are IFN-inducible and have been demonstrated to directly target viral sequences, while others target host mRNAs that encode proteins involved in virus replication, host-virus interactions, signaling, and immune responses (Buggele and Horvath, 2013; Buggele et al., 2013; Gao et al., 2013; Sisk et al., 2013; Zhang et al., 2013; Terrier et al., 2013; Smith et al., 2012; Pedersen et al., 2007; Triboulet et al., 2007). Therefore, the IFN response may potentially use cellular miRNAs as RNA-based antiviral effectors in addition to a myriad of well-known antiviral proteins.

In conclusion, our results suggest that miR-462/731 are involved in IFN-mediated antiviral defense in teleost fish and may partly account for the protective effects of poly I:C and possibly also the early innate protection mediated by the rhabdovirus G-based DNA vaccines. Validated targets of these two miRNAs are currently unknown and whether they can directly target VHSV sequences needs further investigation. To our knowledge, this is the first report to address expression of specific miRNAs in response to DNA vaccination against a fish rhabdovirus and to identify IFN-induced miRNAs that may be involved in host-virus interactions and immune responses in teleost fish. We speculate that miRNA-mediated RNA interference might play an important role in innate antiviral immunity in this highly diverse vertebrate group. Our data may add to a

better understanding of the protective immune mechanisms elicited by fish rhabdovirus DNA vaccines in fish and how these mechanisms may be regulated.

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Figures.

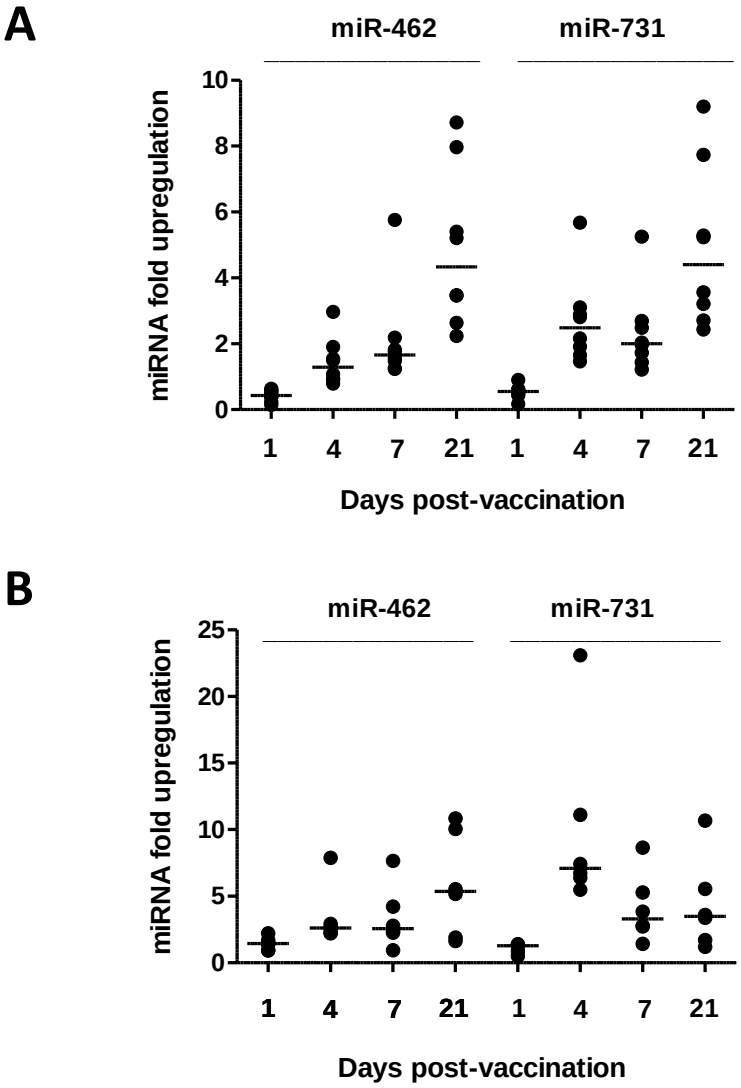

Figure 1. Expression of miR-462 and miR-731 in the skeletal muscle (site of vaccine administration) (A) and in the liver (B) of pcDNA3-vhsG-vaccinated fish at different time points post-vaccination determined by quantitative PCR. Fold upregulation values were calculated in 6 vaccinates relative to the average of 6 plasmid backbone-injected fish. Horizontal bars indicate median values.

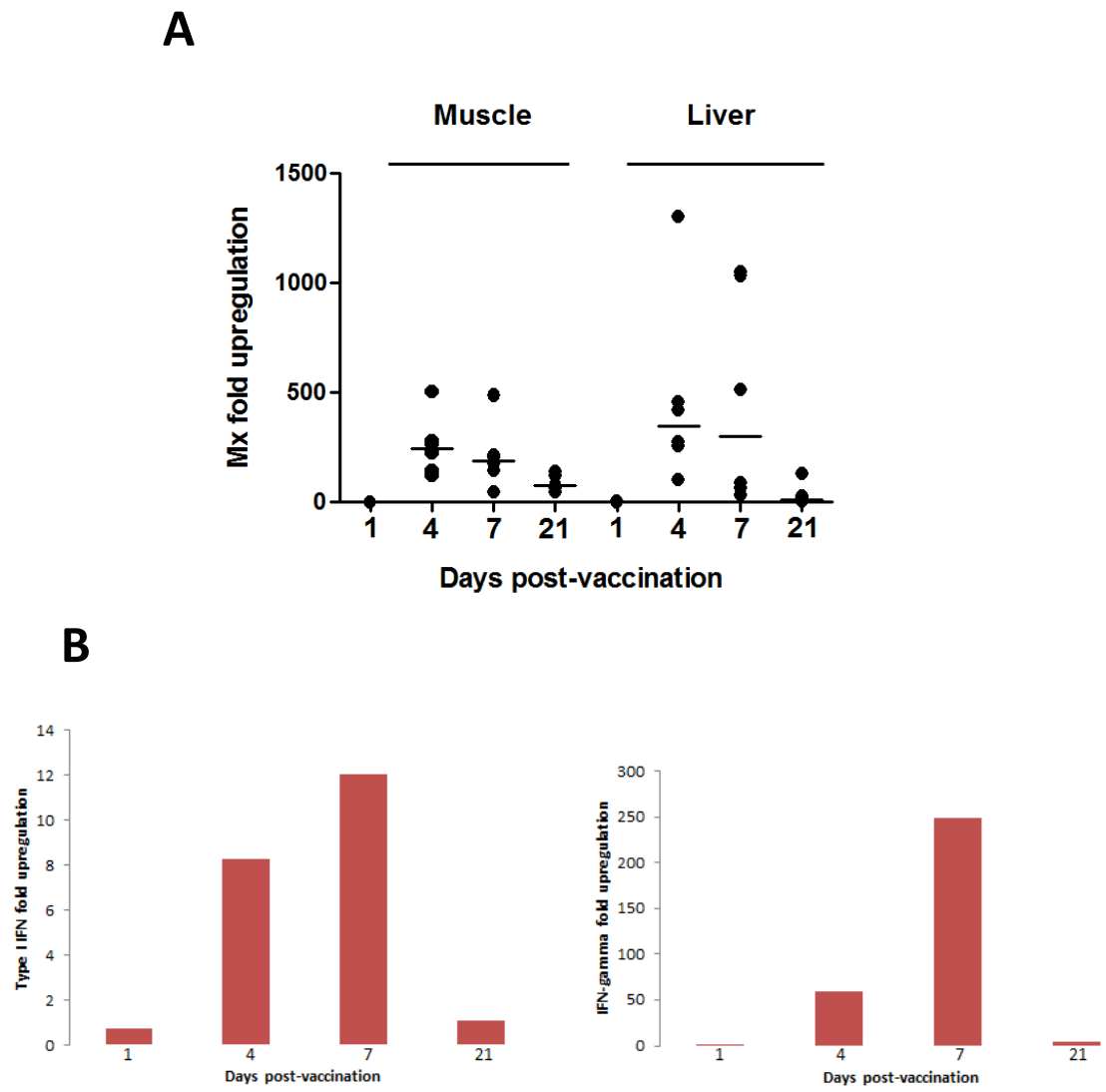

Figure 2. (A) Expression of Mx in the skeletal muscle (site of vaccination) and in the liver of pcDNA3-vhsG-vaccinated fish at different time points post-vaccination determined by quantitative PCR. Fold upregulation values were calculated in 6 vaccinates relative to the mean value of 6 plasmid backbone-injected fish. Horizontal bars indicate median values. (B) Type I IFN and IFN-γ expression at the skeletal muscle (site of vaccination) of pcDNA3-vhsG-vaccinated fish at different time points post-vaccination. Fold upregulation values were measured in a pool of tissue from 6 vaccinated fish relative to a pool of tissue from 6 plasmid backbone-injected fish.

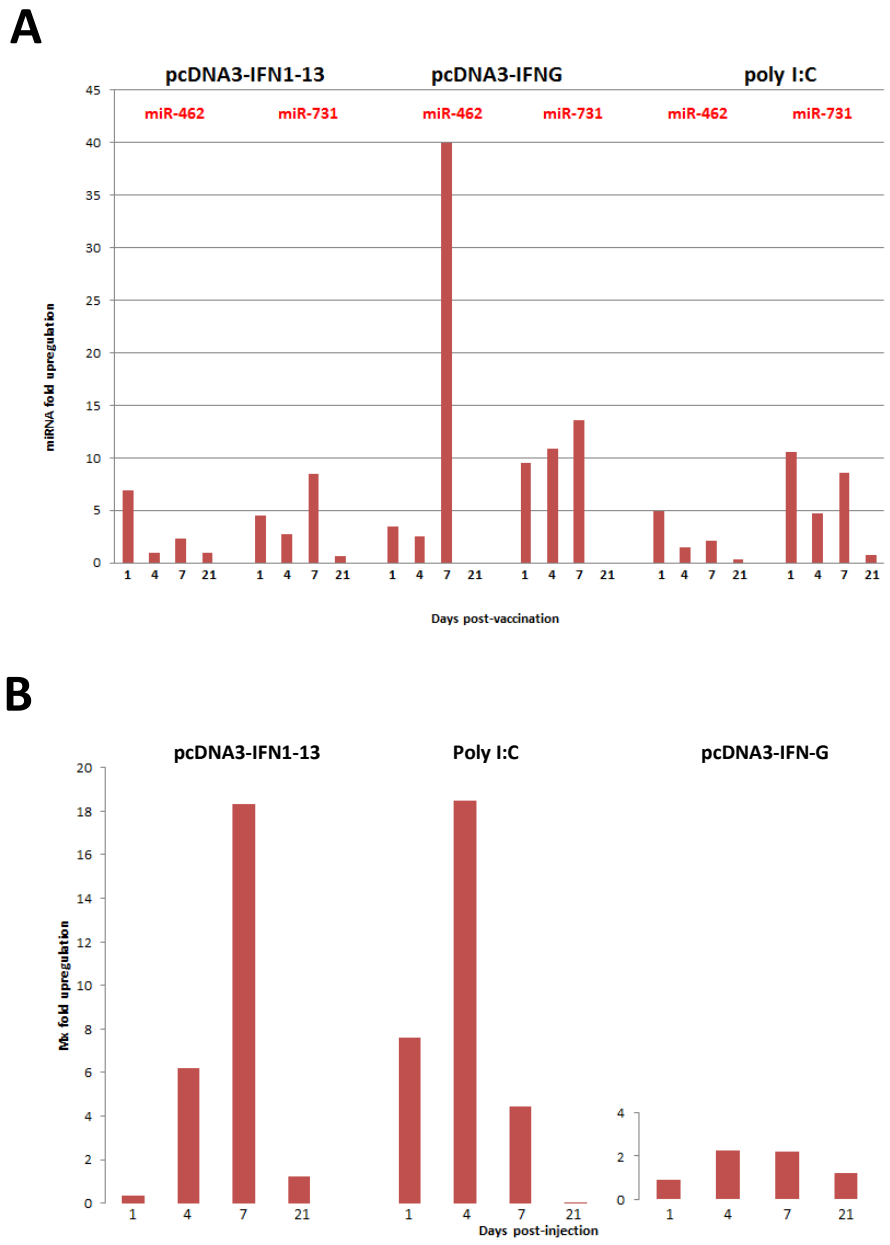

Figure 3. Expression of (A) miR-462 and miR-731 and (B) Mx in the skeletal muscle of pcDNA3-IFN1-13 (type I IFN)-, pcDNA3-IFN-G (IFN-γ)-, and poly I:C-injected fish at different time points post-injection determined by quantitative PCR. Fold upregulation values were measured in a pool of tissue from 6 vaccinated fish relative to a pool of tissue from 6 plasmid backbone-injected fish.

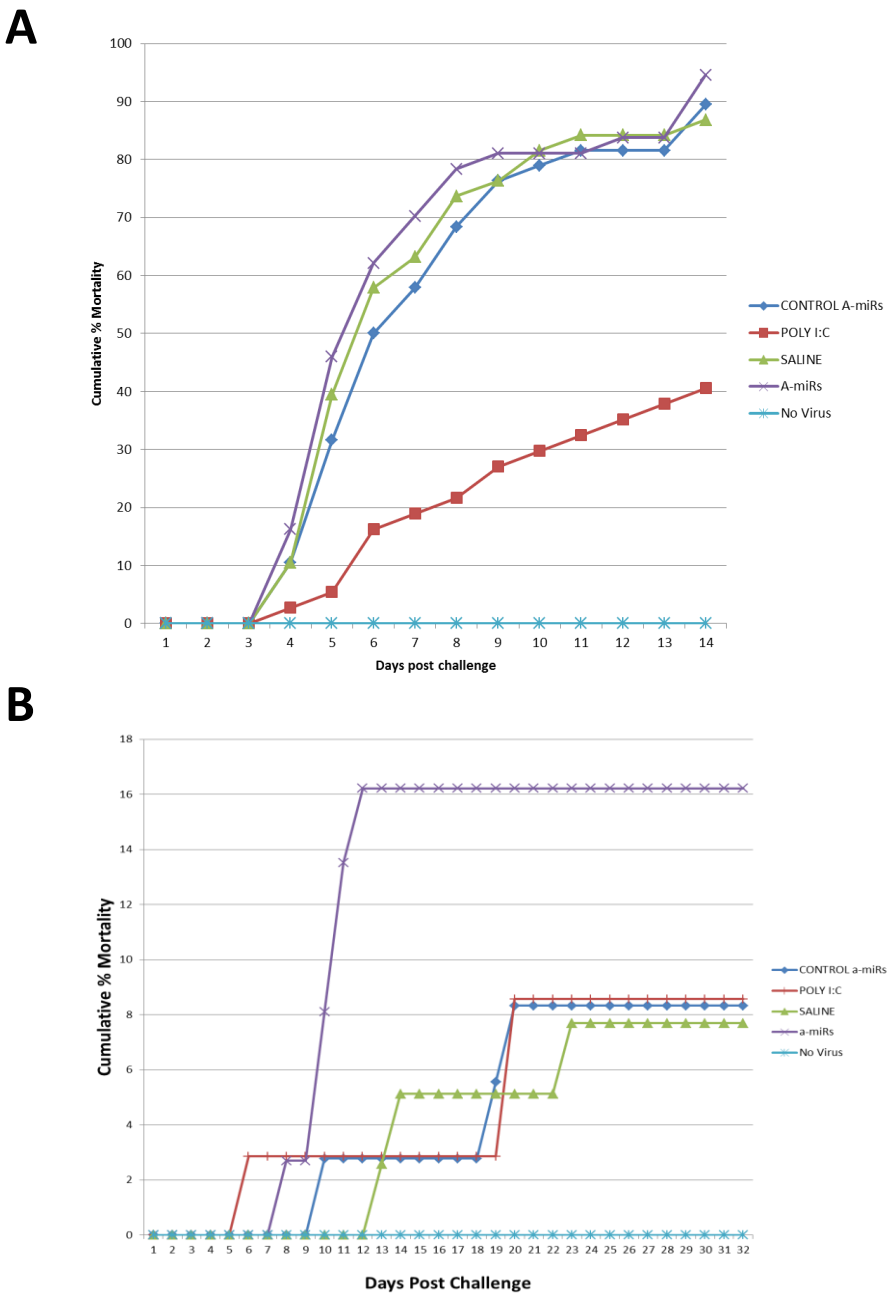

Figure 4. Development of mortality in fish injected with anti-miR-462/anti-miR-731 oligonucleotides followed by challenge with a highly virulent VHSV strain (A) or with a semi-virulent VHSV isolate (B).

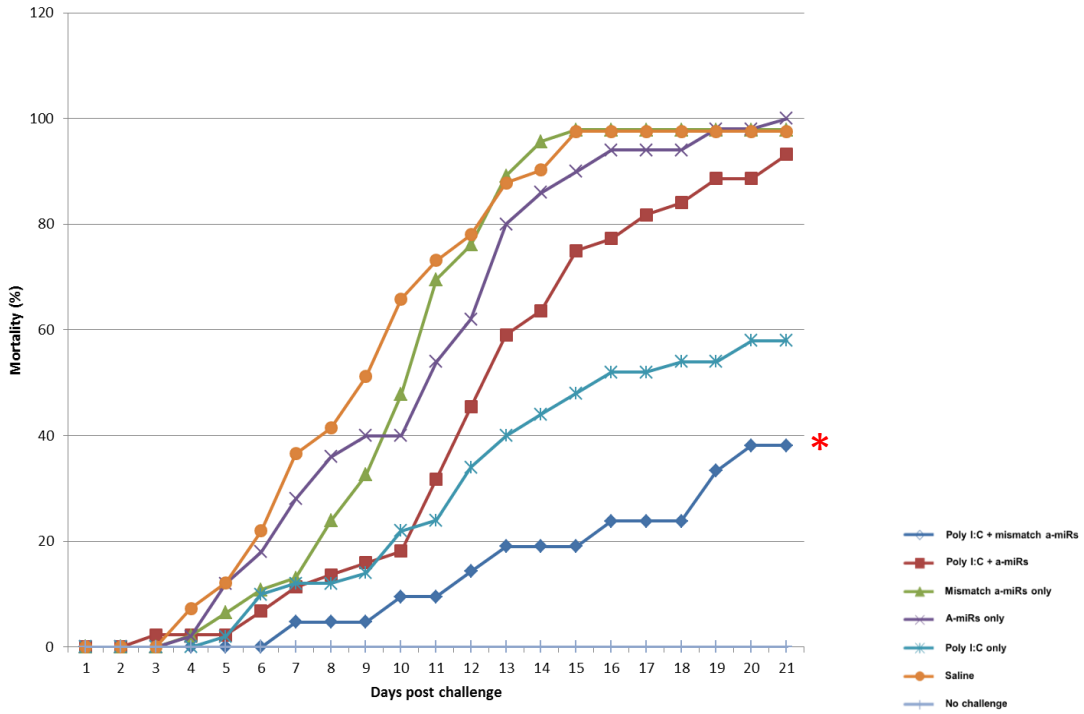

Figure 5. Development of mortality in poly I:C-treated fish in which miR-462 and miR-731 were inhibited using anti-miRNA-specific oligonucleotides and subsequently challenged with a highly virulent VHSV strain. Mortality (%) data are presented as mean values from two replicate aquaria, except for the poly I:C+mismatch a-miR group (*). In this group (*), data from only one aquarium were considered because of the failure of virus challenge in the other replicate aquarium.

MANUSCRIPT 3.

RNA interference in the antiviral defense in vertebrates

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To be submitted.

RNA INTERFERENCE IN THE ANTIVIRAL DEFENSE IN VERTEBRATES

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Abstract

RNA interference (RNAi) is an evolutionarily conserved RNA-mediated cellular process that effectively and specifically inhibits gene expression. Small double-stranded (ds) RNA molecules with complementarity to mRNA mediate translational inhibition or mRNA degradation. Different types of interfering RNAs have been described, the most well-known being the 22-nucleotide (nt) small interfering RNAs (siRNAs) with full sequence complementarity to their target mRNA and microRNAs (miRNAs) of similar size in their mature form, but only complementary to their targets in the 7-nt seed region. In invertebrates and plants, siRNAs are important players in the antiviral defense and are generated by direct processing of the exogenous ds viral RNA. However, in vertebrates, viral dsRNA is recognized by pattern recognition receptors which typically activate the IFN system with its broad range of innate antiviral mechanisms. Interestingly, accumulating data suggests that this includes IFN induced endogenous miRs. Recent reports further revealed that Dicer-processed small RNAs derived from viruses can act as small interfering RNAs (siRNAs) to inhibit complementary viral RNA also in vertebrates. This review gives an update on our current knowledge of RNAi mechanisms involved in antiviral defense in vertebrates.

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Introduction

RNA interference (RNAi) is an evolutionarily conserved phenomenon in which the expression of genes is potently knocked down by an RNA-induced degradation or silencing of homologous mRNAs in the cytoplasm of eukaryotic cells (Meister & Tuschl, 2004; Sharp, 2001). This mechanism posttranscriptionally regulates endogenous gene expression but apart from this regulatory role, RNAi also actively participates in antiviral defense in fungi, invertebrates, and plants (Guo et al., 2012; Han et al., 2011; Ding, 2010; Lu et al., 2005; Segers et al., 2005; Wilkins et al., 2005; Baulcombe, 2004); Hamilton and Baulcombe, 1999).

Viruses exist in almost all ecosystems and are the most abundant infectious agents on Earth. Therefore, organisms have evolved mechanisms to detect viruses and resist infection. Whereas RNAi-based viral RNA silencing plays a central role as an antiviral defense strategy in invertebrates and plants, the interferon (IFN) system is believed to have supplanted RNAi in vertebrates where it predominates as the chief initial antiviral protective mechanism (Umbach and Cullen, 2009); Cullen 2006). The IFN response is elicited by the recognition of virus components such as dsRNA among other pathogen-associated molecular patterns (PAMPs) by the host cells' pattern recognition receptors (PRRs) (Akira et al., 2006). Type I IFNs induce the cells' protein-based antiviral defenses that include the myxovirus resistance (Mx) GTPases, adenosine deaminase (ADA), protein kinase R (PKR)/EIF2AK2, and the 2'-5' oligoadenylate synthetase (OAS)/RNAse L system, upregulation of MHC class I, and apoptotic pathways, among others (Schoggins and Rice, 2011; Borden et al., 2007; Samuel 2001). Induction of the IFN response results in cellular RNA degradation, global shutdown of protein synthesis, and the death of the virus-infected cells (Pichlmair and Reis e Sousa, 2007). During the last decade, it has become evident that RNAi also contributes to antiviral defense

mechanisms in mammals in the form of endogenously coded vertebrate microribonucleic acids (miRNAs). Some of these miRNAs are IFN-inducible and directly target viral transcripts. New data from analysis of the miRNA response to virus infection in teleost fish further suggests that otherwise conserved miRNAs have evolved into IFN-inducible antiviral defense elements in this primitive branch of vertebrates. Finally, the existence of a functional RNAi pathway that directly inhibits viruses in mammalian cells was recently reported in two recent papers showing that small RNAs derived from degradation of viral ds RNA function as siRNAs and silence complementary viral RNA. The implications for our understanding of the role of RNAi in imunnity to viruses are discussed.

The RNAi pathway: gene silencing with siRNAs and miRNAs

Naturally occurring small non-coding RNA effectors of RNAi include siRNAs and miRNAs. SiRNAs originate from the processing of long dsRNA precursors generated by duplex formation of overlapping complementary transcripts (e.g. from repetitive sequences) or may, in some organisms, be generated by RNA-dependent RNA polymerase action on ssRNA templates (e.g. from viral genomes or transcripts) (Meister and Tuschl, 2004). On the other hand, miRNAs are encoded by endogenous genes from which they are transcribed, producing transcripts (primary miRNA/pri-miRNA) containing complementary inverted repeats that fold back on themselves to form hairpin loop structures (Ambros, 2004; Bartel, 2004; Lee et al., 2004).

Small RNAs mature through the activities of the RNAse III-type endonucleases Drosha and Dicer. (Drosha is particularly used to process pri-miRNAs but not long dsRNA precursors of siRNAs) (Meister and Tuschl, 2004).

Pri-miRNAs are processed in the nucleus by Drosha and its co-factor Pasha (DiGeorge Syndrome Critical Region 8 Protein, DGCR8) into stem-loop structures called pre-miRNAs (Gregory et al., 2004; Lee et al., 2003). The pre-miRNAs are transported from the nucleus into the cytoplasm by Exportin 5 (Bohnsack et al., 2004), where they are further processed by Dicer together with TRBP (Tar RNA binding protein) to generate ~22-bp dsRNAs (Skalsky and Cullen, 2010). Long dsRNAs are processed like pre-miRNAs by Dicer into 21-24 bp siRNAs (Meister and Tuschl, 2004).

The RNAi pathway is triggered by Dicer cropping of dsRNA precursors into short siRNA and miRNA duplexes, which despite their different origins, both can be incorporated into the RNA-induced silencing complex (RISC) (Ding, 2010; Zamore et al., 2000; Bernstein et al., 2001). Upon integration into the RISC of one of the two strands (the guide strand), the single-stranded (ss) mature siRNA/miRNA guides the RISC complex to destroy matching or near-matching target mRNAs (Hammond et al., 2000). Complementary sites are usually located in the 3' untranslated regions (UTRs) of target mRNAs and matching between the miRNA seed region (nucleotides 2-7 or 8) and the target mRNA results in mRNA degradation and/or translational inhibition/repression (Bartel, 2009, 2004; Ambros 2004). The siRNAs usually guide RISC to bind perfectly complementary target sites, resulting in mRNA cleavage and degradation, whereas miRNAs typically bind to imperfectly matched targets, causing translational repression. Cases in which siRNAs and miRNAs shift roles with respect to the degree of complementarity of binding site recognition do occur (Meister and Tuschl, 2004). MiRNA-mediated gene silencing by RNAi is acknowledged as a crucial modulator of the expression of genes involved various biological activities in eukaryotes (Bushati and Cohen, 2007; Bartel, 2004). The RNAi pathway is schematically outlined in Figure 1.

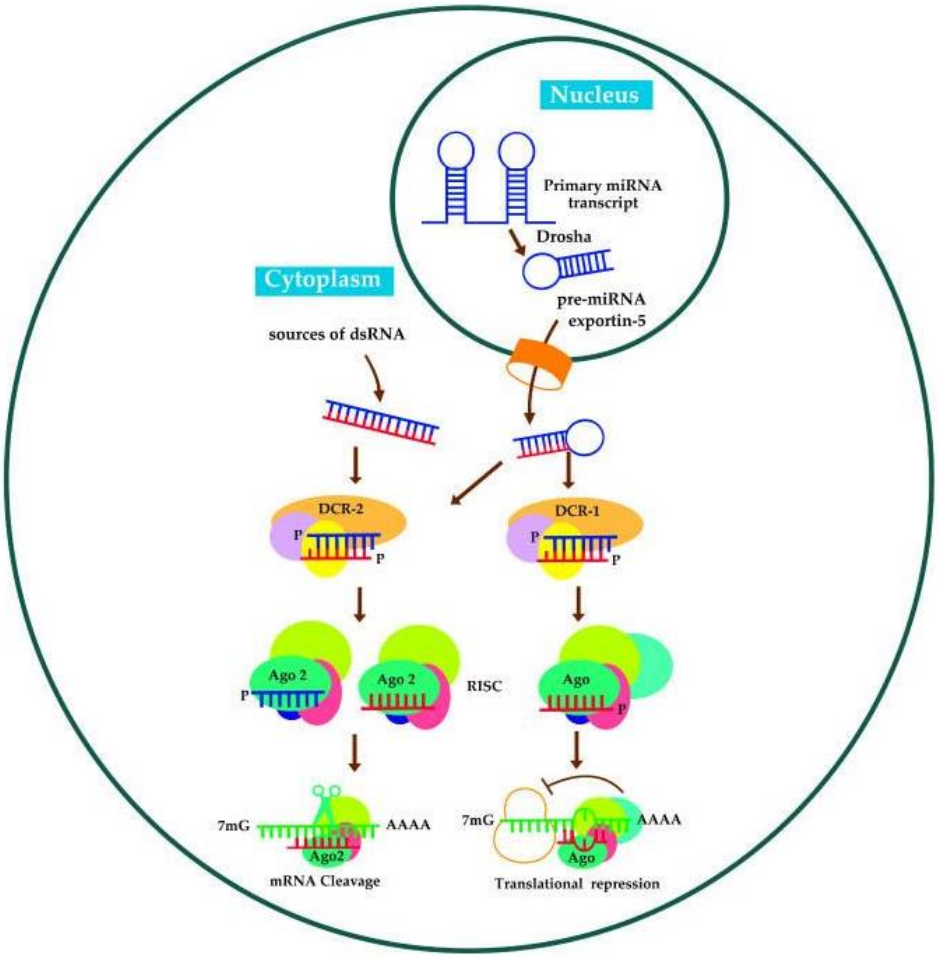

Figure 1. The RNAi pathway. In RNAi, small RNAs guide the regulation of gene expression at the post-transcriptional level. Inside the nucleus, Drosha processes primary miRNA transcripts into precursor miRNA (pre-miRNA). The pre-miRNA is then exported to the cytoplasm through the export receptor exportin-5. Dicer cleaves the pre-miRNA to siRNA-duplexes. The strands of the dsRNA intermediate are separated during incorporation into RISC. The mature miRNAs bound to Argonaute (Ago) proteins guide the RISC to find matching target mRNAs for cleavage or translational suppression. Long dsRNA from exogenous artificial dsRNA, RNA-dependent RNA polymerase (RdRP)-generated dsRNAs, genomic sense and antisense transcripts, and viral RNAs are processed like pre-miRNAs by Dicer into 21–23 nt duplexes. dsRNA stability and Dicer recognition can be regulated by adenosine deaminases (ADARs) and the exonuclease ERI-1. The siRNA-containing RISC is formed with the aid from the RNA helicase Armitage and R2D2. DCR-1 and DCR-2 are two paralogues of Dicer in *Drosophila*; DCR-1 specially cuts pre-miRNAs, while DCR-2 processes long dsRNA. Ago is involved in miRNA-mediated gene silencing, while Ago2 is involved in silencing guided by siRNAs. (Modified from Meister and Tuschl, 2004)

RNAi as antiviral defense mechanism in plants and invertebrates

In fungi, insects, nematodes, plants, and vertebrates, the central role of RNAi in antiviral defense is well characterized (reviewed by Karlikow et al., 2014; Agius et al., 2012; Ding, 2010; Umbach and Cullen, 2009; Ding and Voinnet, 2007) (Figure 2). In these organisms, virus infection elicits the production of virus-derived small RNAs, i.e. siRNAs that mediate antiviral RNAi.

During the replication of all RNA viruses (excluding retroviruses), viral RNA-dependent RNA polymerase (RdRP) produces long, perfectly complementary RNA duplexes as replication intermediates. DsRNA replication intermediates are PAMPs detected and bound by the PRR Dicer that then crops virus dsRNAs into siRNAs that guide RISC to target complementary mRNAs. Viral genomes also contain imperfect hairpins that can serve as substrates for Dicer activity (Bronkhorst et al., 2013; tenOever, 2013). One strand of the siRNA duplex gets loaded into the RISC complex and guides RISC to seek out and bind complementary target viral mRNAs and genomic and antigenomic viral RNAs, resulting in viral RNA degradation (Ding, 2010; Carthew and Sontheimer 2009).

DNA viruses may produce dsRNAs by forming secondary structure in their transcripts or through sense-antisense pairing of transcripts generated by convergent or bidirectional transcription of the genome (Kemp et al., 2013; Bronkhorst et al., 2012; Ding and Voinnet, 2007). In this way, substrates for the generation of virus-derived siRNAs (vsiRNAs) for RNAi are generated and may play a role in the defense against plant DNA viruses (Moissiard and Voinnet, 2006; Blevins et al., 2006). In mammals, dsRNA generation from DNA viruses also occurs since TLR3, a dsRNA sensor, is activated during infection with dsDNA herpesviruses (Zhang et al., 2007; Tabeta et al., 2004).

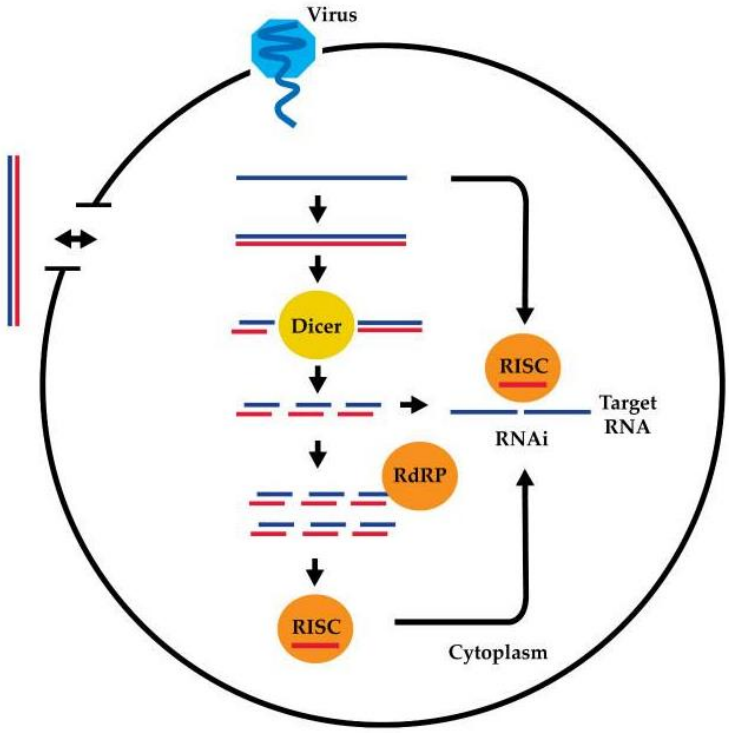

Figure 2. The siRNA-guided antiviral RNAi pathway is elicited by long, perfectly complementary dsRNAs generated during viral replication. These virus-derived dsRNAs are processed by Dicer to generate siRNAs, one strand of which is loaded into RISC either directly or after being used as template by cellular RdRP to generate more dsRNAs, thus amplifying the silencing effects. Virus-derived siRNAs associated with RISC bind target viral mRNAs, resulting in transcript degradation. In plants and invertebrates, virus-derived dsRNAs may be released from virus-infected cells, which prompts a systemic antiviral state after uptake by other non-infected cells (Modified from Umbach and Cullen, 2009).

In some species of plants and invertebrates, Dicer-generated siRNAs can be used as template by cellular RdRP to generate more dsRNAs, thereby augmenting siRNAs that could magnify silencing activity. Virus-derived dsRNAs may subsequently be released from virus-infected cells, initiating a systemic antiviral state following dsRNA uptake by neighboring or distantly located non-infected cells. Systemic RNAi involves the import of dsRNA from the extracellular environment and the systemic and long-distance spread through the whole organism (non-cell-autonomous RNAi) and its progeny (Chapman and Carrington, 2007; Jose and Hunter, 2007; Whangbo and Hunter, 2008).

The highly specific recognition of target viral sequences and the ability to spread systemically exhibits certain parallels with vertebrate adaptive immunity which is based on the specific recognition of antigenic epitopes followed by systemic release of antibodies (Ding, 2010).

As counter defense mechanism, plant and animal viruses are known to encode a wide spectrum of factors (called viral suppresors of RNA silencing or VSRs) and strategies that suppress RNAi (reviewed by Wu et al., 2010). These include VSRs that target protein (e.g. Ago) as well as RNA components of the RNAi machinery (Wu et al., 2010). For example, tombusviruses produce P19 protein that binds and sequesters siRNA duplexes, preventing duplex unwinding and RISC loading. Some viruses encode proteins that recruit endogenous RNAi regulators. Others produce defective interfering RNAs that lack silencing targets (reviewed by Voinnet, 2005). All these reflect the unending evolutionary arms race waged by pathogens and hosts and underscores the crucial function of RNAi and its effectiveness as an antiviral defense strategy.

Antiviral RNAi mechanisms in vertebrates

As had been shown for many viruses infecting invertebrates and plants, vertebrate viruses have also been found to possess components that could help them elude RNA silencing (Li and Ding, 2006). For example, influenza and vaccinia viruses produce IFN antagonist proteins that suppress RNAi (Li et al., 2004), whereas an adenovirus expresses a non-coding RNA (ncRNA) that inhibits the biogenesis of siRNA and miRNA (Lu and Cullen 2004). This indicates an RNAi pathway that may operate against viruses, although the exact role of these VSRs in an antiviral response in mammalian cells remains to be elucidated. Experiments showing the rescue of mutant

viruses lacking a functional VSR in RNAi-defective cells are required to support this (Wu et al., 2010).

Furthermore, various mammalian-infecting viruses are known to be inhibited by synthetic siRNAs in experimental RNAi systems (Haasnoot et al., 2007; Saksela, 2003). Thus, the antiviral prophylactic and therapeutic potential afforded by exogenous siRNAs have been explored in mammalian animal models and cell lines and in humans (DeVincenzo, 2012; Barik, 2010; Morris and Rossi, 2006; Ge et al., 2004; Tompkins et al., 2004). One clinical trial has provided a first proof of concept of the effectiveness of an RNAi-based therapy against a respiratory virus (Alvarez et al., 2009).

However, virus-derived siRNAs (vsiRNAs) have been found either at extremely low levels or are not detectable in mammalian somatic cells infected with a broad spectrum of viruses (Donaszi-Ivanov et al., 2013; Parameswaran et al., 2010; Otsuka et al., 2007; Pfeffer et al., 2004). Sindbis virus (SINV) infection is known to be controlled by RNAi in insects, but no dicer-generated SINV-derived siRNAs can be identified in SINV-infected human cells (Donaszi-Ivanov et al., 2013).

Thus, whether mammalian cells (and potentially vertebrates in general) employ the natural vsiRNA-mediated antiviral RNAi pathway to make cells immune against the virus as do invertebrates and plants, has remained questionable (Umbach and Cullen, 2009). This is partly because in mammalian cells, viral dsRNA triggers the IFN response (Akira et al., 2006; Karpala et al., 2005). It is believed that mammalian cells have replaced the RNAi pathway with the potent IFN system as the predominant protective innate antiviral mechanism (Obbard et al., 2009; Umbach and Cullen, 2009); Cullen 2006) and may conceal the effects of the presumed RNAi response provoked by virus infection.

Natural antiviral virus-derived siRNA-based RNAi operates in mammalian cells. It was only very recently that strong evidence and data have been provided to substantiate the existence of a natural RNAi-based mechanism that (as in invertebrates and plant) relies on siRNA generation from viral dsRNA, targets viral transcripts, and affords protection in vertebrates, particularly in mammalian cells (Maillard et al., 2013) and in suckling mice (Li et al., 2013).

Maillard and co-workers employed undifferentiated, pluripotent mouse embryonic stem cells (mESCs) which are able to survive complete genetic deletion of Dicer and Ago and lack an innate immune response. In encephalomyocarditis virus (EMCV)-infected mESCs, virus-derived small RNAs (vsRNAs) incorporate into the RNA RISC loader Argonaute 2 (AGO2) protein and but were not detected in cells deficient in the siRNA-generating enzyme Dicer (Maillard et al., 2013). Furthermore, mESCs were infected with the wild type Nodamura virus (NoV) which encodes the Dicer inhibitor and RNAi suppressor B2 protein. Whereas the NoV mutant (NoVΔB2) that lacks B2 cannot infect mESCs, its replication was rescued by ectopic expression of either B2 or the Ebola virus viral suppressor of RNAi (VSR) protein VP-35, demonstrating that the mutant virus is unable to suppress RNAi and hence cannot replicate and establish infection (Maillard et al., 2013).

On the other hand, wild type NoV caused lethal infection in suckling mice but infection with NoVΔB2 resulted in zero mortality and virus clearance (Li et al., 2013). Similar innate immune gene expression profiles in mice infected with either the wild type or the mutant NoV indicated that virus clearance was due to the RNAi response rather than the innate immune reactions. vsRNAs with siRNA characteristics accumulated in mice infected with the mutant NoV but not with the wild type virus. Therefore, 7-day-old suckling mice lacking IFN responses are able to mount a strong

antiviral RNAi response, preventing infection by viruses that are unable to suppress the RNAi system. However, wild type NoV failed to infect adult mice despite having a functional RNAi suppressor protein and had been cleared by the IFN system instead (Li et al., 2013). Thus, it was confirmed that a natural antiviral RNAi that relies on siRNA generation by Dicer action on dsRNA viral replication intermediates operates in mammals as it does in plants and invertebrates.

***Is siRNA-based antiviral RNAi physiologically relevant in mammalian cells*?** Despite the compelling evidence presented in favor of an antiviral function for RNAi in mammalian cells and mice, the physiological relevance of siRNA-mediated RNAi antiviral response observed by Maillard and co-workers and Li and co-workers has been disputed (Cullen et al., 2013).

VSR deficiency resulted in virus inhibition by RNAi, strongly emphasizing the crucial function of VSRs. Cullen and co-authors (2013) argued that the evidence substantiating the functional significance of virus-derived siRNAs both in cells and in mice depended exclusively on NoV VSR and on the ability of these vsRNAs to trigger an antiviral RNAi response. Cullen et al. (2013) contended that a more convincing data would have been the demonstration of the rescue of VSR-deficient mutant virus in RNAi-deficient mammalian cells "but not by mutations that inactivate dsRNA-induced IFN responses in mice".

Other authors (Tanguy and Miska, 2013) reasoned that mammals as accidental hosts of the arthropod-vectored NoV are an epidemiological dead-end, thus do not provide the selection pressure for the maintenance of functional VSRs, unlike the arthropods serving as natural NoV reservoirs. Therefore, animal studies using viruses with only mammals as natural hosts are warranted (Tanguy and Miska, 2013).

Results obtained in the two studies also directly contradicted those of one previous study in which IFN-deficient mice infected with a VSR-deficient flu virus succumbed to infection and died (Garcia-Sastre et al., 1998). In the absence of IFN, a virus unable to suppress RNAi would not have been able to cause infection had a functional RNAi been operating.

Furthermore, at around the same time that the papers of Maillard et al. (2013) and Li et al. (2013) came out, one study reported that type I IFN response activation upon virus infection in somatic cells reduced the antiviral action of the RNAi pathway (Seo et al., 2013). The type I IFN antiviral constituents RIG-1/MAVS and RNase L have been shown to contribute to the repression of RISC. In non-infected cells, ISG expression is inhibited by miRNAs, whereas virus detection by PRRs reduces RISC activity leading to heightened ISG expression that increases the protective effect of the IFN response. Therefore, antiviral signaling and RNAi pathways appear to regulate each other's activities and unlike its direct role as an antiviral strategy in invertebrates and plants, RNAi acts as an inhibitor of the type I IFN response (Seo et al., 2013). Cullen and co-authors contend that such reciprocal regulatory activities cannot co-exist if RNAi functions as an authentic antiviral mechanism in mammalian somatic cells (Cullen et al., 2013). In addition, RISC is still inactive at 8 hr post-infection *in vivo* (Seo et al., 2013). Hence, the RNAi machinery cannot yet fight the virus at this point of infection, which opposes the idea that RNAi operates as a crucial defense strategy against virus infection (Cullen et al., 2013).

Interestingly, differentiation of mESCs resulted in decreased siRNA generation from viral dsRNA (Maillard, et al., 2013), whereas antiviral RNAi activity was observed in suckling mice (Li et al., 2013). Therefore it appears that antiviral RNAi acts only in certain cell or tissue types in mammals. The mechanism underlying the loss of antiviral

RNAi in differentiation cells needs to be elucidated. It would seem that RNAi is more relevant as an antiviral defense mechanism in pluripotent stem cells (in which the IFN response is not yet active) because it does not entail apoptosis of infected cells as the IFN response does.

RNA-based antiviral response mediated by cellular miRNAs in vertebrate cells

Because the existence of the invertebrate and plant analogue of a vsiRNA-based antiviral immunity in vertebrate cells has long eluded scientists, it was believed that endogenous miRNAs are instead engaged as primary effectors of viral silencing in an RNAi-based defense (Voinnet, 2005). Unlike the well-elucidated role for siRNAs, the role played by cellular miRNAs in antiviral immunity has remained unclear. Emerging data show that apart from regulating the expression of host genes, some cellular miRNAs also regulate the expression of mammalian virus genes.

The importance of RNAi as a defense strategy against virus infection in mammalian cells is further suggested by observations that impaired RNAi activity boosts viral replication in infected cells. In the absence of Dicer or Drosha, no small RNA effectors that guide the RISC machinery are generated. Accordingly, Drosha- or Dicer-deficiency in mice (Otsuka et al., 2007) or in cultured cells (Triboulet et al., 2007) prevented miRNA production which correlated with more efficient and faster virus replication and caused increased vulnerability to virus infection. Some mammalian viruses have further been shown to suppress RNAi mechanisms in infected cells. Influenza A virus was thus found to target Dicer at both protein and mRNA levels (Matskevich and Moelling, 2007. It was further shown that HIV-1 actively suppresses the expression of cellular miR-17-5p and miR-20a, which target a cellular mRNA whose protein product is used by HIV-1 to its advantage (Triboulet et al., 2007).

Cellular miRNAs that directly target viruses. Working on mammalian cells infected with the primate-infecting retrovirus, *Primate foamy virus* 1 (PFV-1), Lecellier and collaborators (Lecellier et al., 2005) did not detect virus- derived small RNAs. Instead, they found that a cellular miRNA, miR-32, inhibits PFV-1 replication. Silencing miR-32 with LNA inhibitors resulted in accelerated viral replication rates. The PFV-1 genome was further shown to contain potential target sites for miR-32 and for a number of other human miRNAs. In addition, PFV-1 makes an RNAi inhibitor called Tas protein that circumvents the antiviral capability of the RNA silencing pathway and further underscores the importance of this pathway in antiviral defense (Lecellier et al., 2005). Therefore, mammalian cells employ cellular miRNAs rather than take advantage of the virus' own RNA in antiviral RNAi. Lecellier et al. (2005) demonstrated the antiviral effect of a human cellular miRNA against a primate virus. Nevertheless, whereas the ability of PFV-1 to elicit a similar RNA silencing response required further work in a primate cell context, this study was the first to report that a mammalian miRNA possesses direct antiviral activity.

More recently, an increasing number of vertebrate miRNAs have been reported to directly impact gene expression of diverse viruses that infect humans, mice, pig, and chicken (see also review by Russo and Potenza, 2011). Table 1 presents those vertebrate miRNA whose targets in virus genes have been validated experimentally.

Some of these miRNAs target different virus species in the same or in different vertebrate hosts (Table 1). It is theoretically possible that such miRNAs may target other viruses apart from those that are currently reported and may be shown to have a broad range of target viruses, pending new data and further research. One virus species could be targeted by several miRNAs, which may allow regulation by multiple miRNAs. It would be interesting to know whether each of these miRNAs is sufficient

by itself to inhibit the virus or if they exert additive effects to the overall antiviral activity.

Interestingly, a number of vertebrate miRNAs target DNA viruses (MEV, HBV, HPV, ALV, MCMV) (Table 1). Thus far, antiviral RNAi against DNA viruses using virus-derived siRNAs have been demonstrated (Kemp et al., 2013; Bronkhorst et al., 2012), while miRNA-mediated targeting of transcripts produced by vertebrate DNA virus has yet to be demonstrated (Skalsky and Cullen, 2010).

Moreover, cellular miRNAs target and regulate the replication of retroviruses (HIV-1, PFV-1, SIV) (Table 1). Retroviruses (unlike other RNA viruses) do not generate dsRNA as replication intermediates (which can serve as Dicer substrates), hence virus-derived siRNAs may not be produced. Accordingly, retrovirus-derived siRNAs have not been found in HIV-1- or human T cell leukemia virus type I (HTCLV)-infected cells (Lin and Cullen, 2007; Pfeffer et al., 2005). Cellular miRNAs may thus serve as relevant effectors in a natural RNA-based antiviral strategy in the absence of virus-derived siRNAs from retroviruses infecting mammalian cells.

The data described above collectively indicate that vertebrate miRNA targeting of virus genomes and important virus genes contribute to host antiviral defenses. The silencing of virus genes or genomes by cellular miRNAs acting in concert with the cells' many well-known protein-based antiviral resources most likely result in virus elimination and clearance of infection.

Computational prediction has revealed putative target sites of human cellular miRNAs in virus genomes. One study reported that miRNA target sites are mostly found in genomes of RNA viruses (Watanabe et al., 2007). Another report has shown that potential miRNA target sites are highly conserved in genomes of selected human-

infecting viruses (Russo and Potenza, 2011). For a number of human miRNAs and their putative targets in the genome of the influenza H1N1 virus, it has been proposed that the interaction between cellular miRNA and viral RNA potentially prevents viral genome replication instead of silencing gene expression (Zhang et al., 2013), and this may apply to other viral RNA-targeting miRNAs as well. A publicly-available database called ViTa (http://vita.mbc.nctu.edu.tw/) lists known and putative targets sites of chicken, human, mice, and rat miRNAs in various viral genomes (Hsu et al., 2007). Experimental validation of these potential miRNA-virus genome interactions will reveal the antiviral activities of these cellular miRNAs.

There appears to be a selection pressure for certain viruses to conserve miRNA binding sites in their genomes despite the high mutation rates, particularly of RNA viruses. It has been hypothesized that such sequences may have crucial functions (Russo and Potenza, 2011). For example, the miRNA-targeted sequence in an HCV mRNA codes for amino acids of the polymerase and surface antigen proteins (Potenza et al., 2011). These sequences may also harbor important secondary structures or gene regulatory sequences (Russo and Potenza, 2011).

Conversely, whether miRNA targeting of viruses is an authentic antiviral response in vertebrate cells has been a matter of discussion and arguments against the evolution of cellular miRNAs for the specific purpose of antiviral defense have been put forward. Umbach and Cullen (2009) contended that many viruses seem to have evolved much later than their host species. Next, the high mutation rates of virus polymerases create a pool of virus variants (some containing miRNA binding sequences, others lacking them) which could be difficult for cellular miRNAs to effectively suppress. Furthermore, cellular miRNAs being highly conserved and the virus host range

normally narrow make the odds low for miRNAs especially evolving to target a specific virus (Umbach and Cullen, 2009).

On the other hand, in contrast to the sequence conservation of miRNAs, the high mutation rates in viruses lead to more rapid evolution. Mahajan and co-authors (2009) reasoned that this should have given rise to virus variants devoid of miRNA target sequences as the loss of miRNA target sites will prevent miRNA-mediated virus inhibition and is thus an advantage for the virus. The selective pressure that maintains miRNA target sites in virus genomes, apart from reasons mentioned earlier (Russo and Potenza, 2011), may also be exploited by certain viruses to elude the host immune system and to establish chronic infection, as has been proposed for HCV and other persistently-infecting viruses (Mahajan et al., 2008). It is proposed that the rather "modest" effect of miRNAs in inhibiting HCV gene expression would allow the virus to institute low level of chronic infection. This, together with the nonexistence of miRNA target site-deficient HCV variants and the presence of cellular miRNAs much earlier in evolution than HCV is in line with the hypothesis that slowly evolving cellular miRNAs may be hijacked by HCV (and potentially other persistently-infecting viruses) to survive and persist within the host (Mahajan et al., 2008).

Cellular miRNA-virus RNA interaction does not necessarily lead to virus suppression. HCV has been known to co-opt the human liver-specific miR-122 in order to enhance its own replication (Jopling, 2008). Binding of miR-122 to two conserved adjacent sites in the 5'UTR (as opposed to the typical miRNA binding site in the 3'UTR of target RNAs) of HCV RNA (Jopling 2008; Jopling et al., 2005) stabilizes the genome and increases HCV genome replication (Wilson and Huys, 2013). Specifically, miRNA protects the HCV RNA against exonuclease cleavage (Li et al., 2013; Wilson and Huys, 2013). The interaction between miR-122 and HCV RNA also allows the

internal ribosome entry site (IRES) downstream of the miRNA binding site to direct and stimulate protein synthesis (Conrad and Niepmann, 2013; Wilson and Huys, 2013). It is believed that miR-122 is likely to exert positive regulatory activity on HCV miRNA by hitherto unknown mechanisms. Other potential functions for miR-122, the stages of HCV life cycle impacted by miR-122, and related viruses that may likewise be modulated by miR-122 are discussed by Wilson and Huys (2013). Targeting miR-122 with miR-122 inhibitors has been a promising strategy for anti-HCV therapy (Lanford et al., 2010) and has showed encouraging results in Phase II clinical trials (Janssen et al., 2013).

However, it can be argued that the virus production-enhancing interaction between miR-122 and the HCV genome occurs in an unconventional/non-canonical. As described above, miR-122 binds to sites in the 5' UTR of the HCV RNA, whereas canonical miRNA binding that lead to target silencing occurs at the 3' UTR of the target. HCV appears to have usurped miR-122 for its own advantage by its genome interacting with the miRNA at sites different from the site that results in gene silencing.

Cellular miRNAs that target host factors associated with virus-host interactions and antiviral defense. Some cellular miRNAs may exert antiviral effects not by directly targeting virus sequences but by regulating the expression of host genes whose products play roles in cellular processes associated with immune responses and host-virus interactions. Such cellular miRNAs whose target host mRNAs have been experimentally validated are listed in Table 2.

Some miRNAs like miR-125a-5p, -146a, -181 (Tables 1 and 2) have been shown to target both viral RNA and host factors. Other virus-targeting miRNAs like miR-29a, -125b, -146a, -150, -181, -223 (Table 1) have also been previously shown to target

cellular mRNAs and have likewise been implicated in immune-relevant roles (reviewed by Lindsay, 2007).

Of particular interest is miR-146a, which has been known as an important regulator of inflammatory responses (Taganov et al., 2006). Its expression is induced in human monocytes by microbial LPS and by the pro-inflammatory cytokines TNF-α and IL-1β. It is known to target the mRNAs of TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1). TRAF6 and IRAK1 are important adapters downstream of Toll-like and cytokine receptors, hence acts as a negative regulator of immune signalling, preventing uninhibited inflammatory responses (Taganov et al., 2006). miR-146a has been recently shown to be inducible also by IFNβ in human and macaque macrophages infected with SIV and targeted regions of the SIV genome (Sisk et al., 2013).

In addition to those host mRNA targets that have been confirmed experimentally (Table 2), potential host targets of differentially regulated miRNAs in virus-infected cells have been predicted by computational methods. These include genes that play roles in virus entry and replication and in immune-relevant signaling pathways, including the Jak-STAT pathway, the MAPK signaling pathway, cytokine-receptor interactions, and Fc gamma receptor-mediated endocytosis (Zhang et al., 2013; Buggele et al., 2012; Zhao et al, 2012). Functional validation of these putative targets will shed light on the precise roles of the relevant miRNAs in host-virus interactions.

IFN-induced miRNAs. IFNs are known to modulate expression of some cellular miRNAs. A number of IFN-β-induced miRNAs in human cells have been shown to give significant contribution towards the antiviral properties of IFN-β by targeting *Hepatitis C virus* (HCV) sequences and inhibiting HCV replication in vitro (Pedersen

et al., 2007). The target sequences of such miRNAs were found to be conserved across various HCV genotypes (Pedersen et al., 2007). IFN-β and TNF-α stimulated the expression of miR-9, -29a, -29b, and -146a that target the genome of SIV in macrophages (Sisk et al., 2013). IFNα- and IL-28B-treated human hepatoma cells upregulated the miRNA let-7b, which inhibited HCV replication and protein synthesis (Cheng et al., 2013). In human cells, IFN-β induced miR-129-5p, which directly targets the transcription factor SP1; such targeting resulted in the downregulation of expression of E6 and E7 human papillomavirus oncogenes (Zhang et al., 2013).

MiRNAs may thus be a part of the broad range of the antiviral arsenal of IFNs, adding to the many protein-based effectors induced by IFNs and contributes to its antiviral functions.

RNA-based antiviral mechanisms in teleost fishes?

Developing from invertebrates, teleost fish is in evolutionary terms the first group of jawed vertebrates and also the first animal group known to have IFN and adaptive immune mechanisms as known from higher vertebtates. Teleosts consist of highly divergent species that inhabit different aquatic environments and are exposed to a vast number of water-borne viruses. These make it interesting to study the role of RNAi in the antiviral defense in fish.

It is known that a functional RNAi pathway is present and operates in teleost fish, as evidenced by the crucial involvement of miRNAs in zebrafish development (Giraldez et al., 2006). With a broad range of viruses afflicting different fish species and severely impacting aquaculture, the antiviral therapeutic potential of RNAi (using siRNAs or shRNAs, dsRNAs) has also been explored in teleost fishes (reviewed by Lima et al.,

2013; Schyth, 2008) and thus far have been successfully demonstrated in fish cell cultures (Kim et al., 2012; Bohle et al., 2011; Kim and Kim, 2011).

With the recent demonstration of a functional natural RNAi-based antiviral machinery in mammalian cells (discussed in the previous section), it is now clear that the basic mechanism of the RNAi pathway is conserved in eukaryotic viral infections. It is therefore reasonable to assume that it also broadly operates in other vertebrates, including teleost fishes. However, no teleost miRNAs that directly or indirectly target a virus in infected fish or cells have been identified thus far. Likewise, no natural fish virus-derived siRNAs have been identified.

A putative antiviral RNAi in fish is indicated by the induction of Dicer expression in rare minnow (*Gobiocypris rarus*) (Su et al., 2009) and in grass carp (*Ctenopharyngodon idella*) (Shen et al., 2013) infected with *Grass carp reovirus*. It is likewise suggested by the presence of an RNAi antagonist protein in the fish betanodaviruses *Greasy grouper nervous necrosis virus* (GGNNV) (Fenner et al., 2006) and *Striped jack nervous necrosis virus* (SJNNV) (Iwamoto et al., 2005). Fish betanodavirus protein B2 sequence-independently binds to and sequesters dsRNA, protecting it from cleavage by Dicer and enables the accumulation of viral RNA (Fenner et al., 2006). The B2 protein when mutated to remove amino acids needed to bind dsRNA has decreased its ability to block the silencing of a GFP reporter (Fenner et al., 2007).

Furthermore, a fish homologue of a protein involved in cellular dsRNA uptake (Duxbury et al., 2005; Feinberg and Hunter 2003; Winston et al., 2002) called systemic RNA interference defective protein 1 (SID-1) (called ScSidT2 in mandarin fish, *Siniperca chuatsi*) has been shown to be upregulated in mandarin fish infected with *Infectious spleen and kidney necrosis virus* (ISKNV) (Ren et al., 2011; He et al., 2006)

while antiserum neutralization of this protein results in increased ISKNV replication in cell culture (Ren et al., 2011). When ScSidT2 was overexpressed in fathead minnow (FHM) cells, an increased import of exogenous dsRNA was observed and inhibited tiger frog virus replication in these cells (Ren et al., 2011), indicating a role in antiviral defense in fish cells. The gene encoding SID-1 is conserved from invertebrates to humans (Luo et al., 2013) and SID-1-mediated transport of dsRNA has been implicated in systemic RNAi in *C. elegans* (Calixto et al., 2010; Feinberg and Hunter, 2003; Winston et al., 2002). The presence of this protein in fish may indicate that a systemic RNAi may operate in fish, as had been shown in *C. elegans* and plants, although this phenomenon has not yet been shown to occur in vertebrates.

Research in our lab revealed that two miRNAs, miR-462 and miR-731, currently described only in teleost fish, were strongly upregulated in rainbow trout (*Oncorhynchus mykiss*) infected with *Viral hemorrhagic septicemia virus* (VHSV), a fish rhabdovirus (Schyth et al. manuscript submitted) and in fish following the administration of a DNA vaccine encoding the glycoprotein (G) gene of VHSV (Bela-ong et al., manuscript in preparation). These two IFN-inducible miRNAs have been shown to be orthologues of conserved ancestral miRNAs found in a cartilaginous fish and higher vertebrates. In humans, they are involved in regulation of the cell cycle. Silencing miR-462 and miR-731 with specific anti-miRNAs in poly I:C treated fish reduced the ability of this TLR3 agonist and IFN inducer to protect fish from lethal virus challenge, suggesting their involvement in innate antiviral defense. Whereas these miRNAs are conserved across vertebrates in terms of nucleotide sequence, they appeared to have functionally evolved into IFN-induced antiviral defense elements in the teleost fish (Bela-ong et al. manuscript in preparation; Schyth et al., manuscript submitted). Validated cellular as well as putative viral targets of miR-462 and miR-731

are currently unknown (Bela-ong et al. manuscript in preparation). As with numerous miRNAs in mammalian cells, they may regulate the expression of infection- and defense-associated host factors or may directly target viral genomes or transcripts. Bioinformatic analysis indicates the presence of potential target sites of these two miRNAs in the genome of VHSV. Further studies aimed at characterizing the detailed function of these miRNAs in the context of infection is currently underway.

Teleost fish miRNAs are presently underrepresented in the miRNA repository miRBase (http://mirbase.org) being limited to 8 species, notwithstanding the enormous species diversity and the economic importance of fish in both wild fisheries and aquaculture. These include miRNAs identified in the model species zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) and the aquaculture fish species common carp (*Cyprinus carpio*) and Channel catfish (*Ictalurus punctatus*). A total number of 1250 teleost fish pre-miRNA hairpin sequences and 1044 mature miRNAs are currently listed in mirBase (http://mirbase.org, accessed 22 April 2014) and new teleost miRNAs, some of them fish-specific, are being discovered.

Interestingly, most of miRNAs shown to target viruses or those that have been shown to play roles in antiviral responses in higher vertebrates (mostly in mammals) (listed in Tables 1 and 2) have homologues in teleost fish, at least in zebrafish according to miRbase. Whether these teleost fish homologues perform equivalent functions as their mammalian counterparts remains to be analyzed.

Research will greatly benefit from teleost miRNA discovery and miRNA profiling in virus-infected fish, and functional studies conducted in parallel should pave the way for elucidating potential antiviral RNAi pathways in this highly diverse vertebrate group. This also entails the development of appropriate cell culture and fish experimental

systems (i.e. RNAi-deficient and IFN-deficient cells/fish, VSR-deficient teleost-infecting viruses) and discovery of teleost-infecting viruses encoding potential VSRs and characterization of putative RNAi-blocking activity in teleost systems.

Concluding remarks

RNA-based antiviral response is a conserved mechanism that operates in plants, invertebrates, and vertebrates. In invertebrates and plants, antiviral RNAi uses the virus' own genetic material to create an antiviral weapon in the form of vsiRNAs which are used by the RNAi machinery to destroy the virus' RNAs. Virus-encoded VSRs that inhibit the RNAi machinery and the potent IFN response most likely conceal the effects of antiviral RNAi mechanisms in mammalian cells such that its existence here has gone unnoticed for some time in mammalian cells. Two very recent reports have identified vsiRNAs in mESCs and in suckling mice functioning in antiviral RNAi that appears to operate in a context-dependent manner (i.e. only in undifferentiated cells lacking the IFN response or in specific tissue types).

Emerging data provide strong evidence favoring the antiviral role of cellular miRNAs and their contribution to vertebrate immune defense. Together with IFN-induced effectors, cellular miRNAs may serve in the frontline defense against viruses in vertebrate cells and the list of such will most likely increase.

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Table 1. Vertebrate cellular miRNAs with experimentally validated targets in viral genes.

miRNA	Vertebrate species	Target virus	Target in viral genome	References
let-7b		Hepatitis C virus (HCV)	Conserved coding sequences of NS5B and 5'-UTR of HCV genome	Cheng et al., 2012
miR-9	Human and macaque primary macrophages	Simian immunodeficiency virus (SIV)	Nef/U3 and R regions	Sisk et al., 2013
miR-24	Mice	Vesicular stomatitis virus (VSV)	large (L) protein and phosphoprotein (P)	Otsuka et al., 2007
miR-28	Human	Human immunodeficiency virus 1 (HIV-1)	3' end of RNA	Huang et al., 2007; Reviewed by Russo and Potenza, 2011
miR-29a	Human and macaque primary macrophages	SIV	Nef/U3 and R regions	Sisk et al., 2013
miR-29a	Human	HIV-1	Nef protein coding sequence; 3' UTR of genomic RNA	Nathans et al., 2009; Ahluwalia et al., 2008; Reviewed by Russo and Potenza, 2011
miR-29b	Human and macaque primary macrophages	SIV	Nef/U3 and R regions	Sisk et al., 2013
miR-93	Mice	VSV	large (L) protein and phosphoprotein (P)	Otsuka et al., 2007
miR-125a-5p	Human	Hepatitis B virus (HBV)	Overlapping surface antigen and polymerase ORFs	Potenza et al., 2011; Reviewed by Russo and Potenza, 2011
miR-125a-5p	cultured hepatic cells	HBV	viral polymerase ORF and the overlapping surface antigen ORF	Potenza et al., 2011
miR-125b	Human	HIV-1	3' end of RNA	Huang et al., 2007; Reviewed by Russo and Potenza, 2011
miR-125b	Human	Human papilloma virus	Capsid protein L2 coding sequence	Nuovo et al., 2010; Reviewed by Russo and Potenza, 2011
miR-142-3p	Murine myeloid-lineage cells (RAW 264.7)	North American eastern equine encephalitis virus	3' UTR of RNA genome	Trobaugh et al., 2014
miR-146a	Human and macaque primary macrophages	SIV	Nef/U3 and R regions	Sisk et al., 2013
miR-150	Human	HIV-1	3' end of RNA	Huang et al., 2007; Reviewed by Russo and Potenza, 2011

Table 1. Continued……. Vertebrate cellular miRNAs with experimentally validated targets in viral genes.

miRNA	Vertebrate species	Target virus	Target in viral genome	References
miR-181	Cat (cell line)	Mink enteritis virus (MEV)	NS1 mRNA	Sun et al., 2013
miR-181	monkey kidney cells and pigs	Porcine reproductive and respiratory syndrome virus (PRRSV)	conserved region downstream of ORF4 of viral genome	Guo et al., 2013
miR-196	Human	HCV	Nonstructural protein 5A coding sequence	Pedersen et al., 2007; Hou et al., 2010; Reviewed by Russo and Potenza, 2011
miR-199a-3p	Human	HCV	5'-UTR of genomic RNA	Murakami et al., 2009; Reviewed by Russo and Potenza, 2011
miR-199a-3p	Human	HBV	Overlapping surface antigen and polymerase ORFs	Zhang et al., 2010; Reviewed by Russo and Potenza, 2011
miR-210	Human	HBV	Overlapping surface antigen and polymerase ORFs	Zhang et al., 2010; Reviewed by Russo and Potenza, 2011
miR-223	Human	HIV-1	3'-end of RNA	Huang et al., 2007; Reviewed by Russo and Potenza, 2011
miR-296-5p	Human cells	Enterovirus 71	Sites in EV71 genome	Zheng et al., 2013
miR-323	MDCK cell	Influenza A virus	PB1 gene in virus genome	Song et al., 2010
miR-382	Human	HIV-1	3'-end of RNA	Huang et al., 2007; Reviewed by Russo and Potenza, 2011
miR-448	Human	HCV	Core protein coding sequence	Pedersen et al., 2007; Russo and Potenza, 2011
miR-491	MDCK cells	Influenza A virus	PB1 gene in virus genome	Song et al., 2010
miR-654	MDCK cells	Influenza A virus	PB1 gene in virus genome	Song et al., 2010
miR-1650	Chicken cells line)	Avian leucosis virus	5' UTR of viral genome	Wang et al., 2013
miR-27a and miR-27b	Mice	Murine cytomegalovirus		Buck et al., 2010

Table 2. Vertebrate cellular miRNAs with validated host factor mRNA targets whose protein products affect virus propagation/replication and antiviral responses.

miRNA	Vertebrate species	Infecting virus	Host targets	References
miR-17-5p	Human (PBMCs)	HIV-1	histone acetyltransferase Tat cofactor PCAF	Triboulet et al., 2007
miR-20a	Human (PBMCs)	HIV-1	histone acetyltransferase Tat cofactor PCAF	Triboulet et al., 2007
miR-122	Human	HBV	Cyclin G1	Wang et al., 2012
miR-129-5p	Human (HeLa and cervical cancer cells)	HPV	Transcription factor SP1	Zhang et al., 2013
miR-132	primary lymphatic endothelial cells (LECs)	Kaposi's sarcoma-associated herpesvirus (KSHV)	p300 transcriptional co-activator; ISGs	Lagos et al., 2010
miR-132	monocytes	herpes simplex virus-1 (HSV-1)		Lagos et al., 2010
miR-132	monocytes	human cytomegalovirus (HCMV)		Lagos et al., 2010
miR-146a	Human lung epithelial cells (A549)	Influenza A virus	Multiple targets involved in Toll-like receptor pathway, innate immune response, cytokine production, and apoptosis	Terrier et al., 2013
Hs_154	Human cells (HEK293T and SK-N-MC)	West Nile virus	CCCTC-binding factor (CTCF) and epidermal growth factor receptor (EGFR)-coamplified and overexpressed protein (ECOP/VOPP1)	Smith et al., 2012
miR-181	Pig (blood monocytes and alveolar macrophages	PRRSV	3’ UTR of CD163 mRNA	Gao et al., 2013
miR-196	Human	HCV	3’ UTR of Bach 1 (basic leucine zipper mammalian transcriptional repressor)	Pedersen et al., 2007; Hou et al., 2010; Reviewed by Russo and Potenza, 2011
miR-198	monocytes	HIV-1	cyclin T1	Sung and Rice, 2009
miR-203	Human A549 cells	Sendai virus	IFIT1/ISG56 transcript	Buggele and Horvath, 2013
miR-449b	Human A549 cells	Influenza A virus	histone deacetylase 1 and interferon beta	Buggele et al., 2013